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Investigation of the Shiga toxin 2 B subunit and its immunoprophylactic potential

by

Paola Marcato ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Bacteriology**

Department of Medical Microbiology and Immunology

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Abstract

The Shiga toxins (Stx), especially Stx2, contribute to the development of enterohemorrhagic *Escherichia coli*-mediated colitis and hemolytic-uremic syndrome. The Stx2 B subunit that binds the host cell receptor, globotriaosylceramide (Gb_3), was cloned and expressed by replacing the Stx2 B subunit leader peptide nucleotide coding sequences with those from the Stx1 B subunit. The Stx2 B subunit assembled into a pentamer and bound to synthetic analogs of the Gb_3 receptor. The Stx2 B subunit was not cytotoxic to Burkitt's lymphoma cells, Vero cells, human renal cortical epithelial cells, mesangial cells, renal proximal tubule cells and microvascular endothelial cells.

The immunoprophylactic properties of the cloned Stx2 B were initially investigated in rabbits. Inducing an anti-Stx2 B immune response in rabbits required multiple injections and the incorporation of lipopolysaccharide (LPS). Nevertheless, some rabbits developed an anti-Stx2 B subunit immune response, as determined by immunoblot analysis and cytotoxicity neutralization assays and were protected from a challenge with Stx2 holotoxin.

Further studies were performed to determine conditions for overcoming the hypoimmunogenicity of the Stx2 B subunit. Mice were immunized with recombinant Stx2 B subunit alone or after conjugation to Keyhole limpet hemocyanin (KLH) presented in either Quil-A with or without LPS, the Ribi adjuvant system (RAS) with or without monophosphoryl lipid A (MPL), or

Alhydrogel with or without MPL. The resulting immune responses were then evaluated by assessing serum immunoglobulin titers, immunoblot reactivities, cytotoxicity neutralization and protection from a lethal challenge with Stx2 holotoxin. The studies revealed that the Stx2 B subunit-KLH conjugate, when presented twice in admixture with RAS, induced a robust antigen-specific protective immune response in 100% of the immunized mice.

The cloned Stx2 B subunit was also used to investigate the interaction between Stx2 and human serum amyloid P component (HuSAP). Solid-phase binding, competitive binding, and cytotoxicity neutralization assays indicate that the B pentamer and A subunit are both required and contribute to HuSAP binding to Stx2. Therefore, if the Stx2 B subunit is used immunoprophylactically, it is unlikely that the B subunit alone will interact with HuSAP and, thereby, inhibit its immune response.

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List of Abbreviations

ABTS	2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
A/E	attaching and effacing
ARC	Alberta Research Council
BCA	bicinchoninic acid
Bfp	Bundle forming pili
BSA	bovine serum albumin
CCP	clathrin-coated pits
CCS	cell culture supernatant
CD_{50}	50% cytotoxic dose
CDT	Cytolethal distending toxin
CD-TEC	Cytolethal distending toxin-producing *Escherichia coli*
CFAs	colonization factor antigens
CFU	colony forming unit
CIHR	Canadian Institutes for Health Research
CRP	C-reactive protein
DHEC	Diffuse-adherent *Escherichia coli*
DNA	deoxyribonucleic acid
EAggEC	Enteroaggregative *Escherichia coli*
ECL	enhanced chemiluminescence

EDC	1-Ethyl-3-(-3-Dimethylaminopropyl) carbodiimide hydrochloride
EGM-2-MV	microvascular endothelial cell growth medium-2
EHEC	Enterohemorrhagic *Escherichia coli*
EIA/RIA	enzyme-immunoassay / radioimmunoassay
EIEC	Enteroinvasive *Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EPEC	enteropathogenic *Escherichia coli*
ER	endoplasmic reticulum
Esps	*Escherichia coli* secreted proteins
ETEC	Enterotoxigenic *Escherichia coli*
FBS	fetal bovine serum
FCA	Freud's complete adjuvant
FITC	Fluorescein isothiocyanate
Gb_3	globotriaosylceramide
Gb_4	globotetraosylceramide
HC	hemorrhagic colitis
HMVEC	human microvascular endothelial cells
HPLC	high performance liquid chromatography
HRCE	human renal cortical epithelial cells
HRP	horse radish peroxidase
HUS	hemolytic uremic syndrome

HuCRP	human C-reactive protein
HuSAP	human serum amyloid P component
Hly	hemolysin
ID	identification
Ipas	Invasins
KLH	Keyhole limpet hemocyanin
LAL	limulus amebocyte lysate
LB	Luria-Bertani broth
LD_{100}	100% lethal dose
LEE	locus of enterocyte effacement
LPS	Lipopolysaccharide
LT	Heat-labile enterotoxin
MAP	Mitochondria associated protein
MEM	minimal essential medium
MCO	methoxycarbonyloctyl
MPL	monophosphoryl lipid A
MsGM	mesangial cell growth medium
MTT	(3-[4, 5-dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide
MW	molecular weight
MWCO	molecular weight cut-off
NHMC	normal human mesangial cells

No	number
NT	neutralization titers
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.05% Tween
PCR	polymerase chain reaction
PMN	polymorphonuclear cell
PVDF	polyvinylidene fluoride
QCL	quantitative chromographic limulus amebocyte lysate
RAS	Ribi adjuvant system
REGM	renal epithelial cell growth medium
RPTEC	renal proximal tubule epithelial cells
TDM	trehalose dicorynomycolate
TGN	trans Golgi network
Tir	translocated intimin receptor
TN	10 mM Tris Base (pH 8.0) and 0.14 M NaCl
TTP	thrombotic thrombocytopenic purpura
TTSS	type III secretion system
TUNEL	terminal transferase-mediated dUTP nick end labeling
SAP	serum amyloid P component
SDS	sodium dodecyl sulfate
ST	Heat-stable enterotoxin

STEC	Shiga toxin producing *Escherichia coli*
Stx	Shiga toxin
USP	United States Pharmacopeia
VT	Verotoxin

Chapter 1

General Introduction

1. 1. Enterohemorrhagic *Escherichia coli* (EHEC) and human infection

J. Konowalchuck and colleagues first reported that extracts of *E. coli* serotypes isolated from patients with bloody diarrhea were cytotoxic to African green monkey kidney epithelial (Vero) cells (Konowalchuk et al., 1977). It was later shown that this cytotoxic activity was due to an *E. coli* exotoxin, which was named Verotoxin (VT) (Karmali et al., 1983). Today, the toxins are commonly known as Shiga toxins (Stx) due to their similarity to the toxin produced by *Shigella dysenteriae* whose discovery predates the VT by nearly 100 years (Nataro and Kaper, 1998). The Shiga toxins produced by enterohemorrhagic *Escherichia coli* (EHEC) strains are key virulence factors contributing to the development of hemorrhagic colitis (HC) and, in some cases, the potentially lethal complication of hemolytic-uremic syndrome (HUS).

EHEC represent one of seven currently known enterovirulent groups of *E. coli* that are uniquely designated because of the diseases they cause and distinguishing pathogenic mechanisms (Table 1-1) (Clarke, 2001). EHEC serotypes are further classified from the broader group of Shiga toxin-producing *E. coli* (STEC) that in addition to producing one or more Stx, also cause clinical illness in humans. EHEC also harbor a 90kb virulence plasmid and cause attaching-effacing lesions (described in detail later) (Meng et al., 1998). More than 50 EHEC serotypes

Enterovirulent *E. coli*	**Distinguishing virulence factor(s)**	**Clinical symptoms**
Enterohemorrhagic (EHEC)	Shiga toxins (Stx1/Stx2), Tir, intimin, *E. coli* secreted proteins (Esps), hemolysin, ToxB, Type three secretion system (TTSS)	Hemorrhagic colitis and hemolytic-uremic syndrome (Hamburger disease)
Enteropathogenic (EPEC)	Bundle-forming pili (BFP), intimin, Tir, Esps, TTSS	Infant diarrhea
Enterotoxigenic (ETEC)	Heat-stable (ST)and heat-labile (LT) enterotoxins, colonization factor antigens (CFAs)	Watery diarrhea (Travelers' diarrhea)
Enteroaggregative (EAggEC)	Enteroaggregative heat-stable enterotoxin	Watery mucoid diarrhea
Enteroinvasive (EIEC)	Invasins (Ipas)	Dysentery
Diffuse-adherent (DAHEC)	α-hemolysin, cytotoxic necrotizing factor 1, α-hemolysin	May cause watery mucoid diarrhea, fever, nausea.
Cytolethal distending toxin-producing (CD-TEC)	Cytolethal distending toxin (CDT)	May cause watery diarrhea

Table 1-1. Classes of enterovirulent *E. coli.* Summarized from reviews by S. C. Clarke. and J. P. Nataro & J. B. Kaper (Clarke, 2001; Nataro and Kaper, 1998). The virulence factors listed for EHEC will be described in detail later.

have been isolated from humans suffering from HC, however the most prevalent serotype is O157:H7 (Besser et al., 1999; Bettelheim et al., 2003; Elliott et al., 2001; Tanaka et al., 2002; Tarr, 1995). Table 1-2 compares the presence of virulence markers in EHEC and STEC isolates (Meng et al., 1998). A comprehensive list of STEC serotypes can be found at http://www.microbionet.com.au

The people most at risk in the population for developing the more serious complication of HUS from an EHEC infection are the very young and very old (Carter et al., 1987; Rocchi and Capozzi, 1999; Ryan et al., 1986; Waters et al., 1994; Wood et al., 2001). EHEC infections are zoonotic as cattle and other domestic ruminants act as reservoirs for the organisms (Meng et al., 1998; Renter and Sargeant, 2002; Van Donkersgoed et al., 1999). If humans consume food or water that has come into contact with fecal matter from an EHEC-colonized animal they can develop HC and HUS. Consumption of contaminated under-cooked ground beef, un-pasteurized milk or dairy products produced with un-pasteurized milk, untreated drinking water, un-pasteurized juices, and fruits and vegetables have all been implicated in outbreaks and sporadic cases of EHEC infections (Banatvala et al., 2001; Goh et al., 2002; Govaris et al., 2002; Holme, 2003; Ogden et al., 2001; Olsen et al., 2002; Samadpour et al., 2002; Wachtel et al., 2003; Yamaguchi et al., 2003). Person-to-person spread has also been reported (Slutsker et al., 1998). There is a zero tolerance for the presence of the organisms in the food chain as the infectious dose of EHEC can be as low as 10^1

Presence of EHEC-*hlyA*, *eae*, *stx1* and *stx2* genes in EHEC, other STEC and other *E. coli*

Group	No. of isolates	Serotype[a]	EHEC-*hlyA*	*eae*	*stx1*	*stx2*
E. coli O157:H7	120					
	98		+	+	+	+
	20		+	+	−	+
	2		+	+	+	
Non-O157 EHEC	16					
	1	O-:H11	+	+	+	+
	1	OR:H9	+	+	+	−
	1	O5:NM	+	+	+	
	1	O26:NM	+	+	+	+
	1	O26:H11	+	+	+	
	1	O45:H2	+	+	+	
	1	O50:H7	+	+		+
	1	O68:NM	−	+		+
	1	O103:H2	+	+	+	+
	1	O104:NM	+	+		+
	1	O111:NM	+	+	+	
	1	O113:K75:H21	+	+		+
	1	O125:NM	+	+	+	
	1	O153:H2	+	+	+	+
	2	O157:NM	+	+		+
Non-O157 STEC	15					
	1	U:NM	+	+	+	
	1	O5:NM	+		+	+
	1	O22:H8	+			+
	1	O26:H11	+	+	+	
	1	O46:H38	+		+	+
	1	O88:H49	+		−	+
	1	O91:NM	−		+	
	1	O91:H21	+		+	+
	2	O103:H2	+	+	+	
	1	O111:H8	+	+	+	
	2	O111:H11	+	+	+	
	1	O111:NM		+	+	+
	1	O126:H8		+	+	+
Other pathogenic *E. coli*	26					
	8		−	+	−	
	18					
E. coli K12	1		−		−	
Total	178					

Table 1-2. List of EHEC and STEC serotypes isolated from humans, cattle, sheep and food. This list is not comprehensive as it does not include all known EHEC and STEC serotypes, but does illustrate the differences between the two groups with respect to some key virulence determinates. Each isolate was tested for the presence of certain virulence genes (*hlyA*, *eae*, *stx1* and *stx2*) using polymerase chain reaction with specific primers for each gene. *hlyA* is part of an operon that encodes a hemolysin on the virulence associated plasmid (described in detail later). *eae* encodes intimin, a critical adherence protein (described in detail later) (Meng et al., 1998).

to 10^2 organisms (Bell et al., 1994; Paton et al., 1996; Tilden et al., 1996). The low infectious dose of EHEC also explains the person-to-person spread.

1. 2. EHEC colonization of human intestine and hemorrhagic colitis

EHEC employ colonization and pathogenic strategies similar to those of enteropathogenic *E. coli* (EPEC, a common cause of infant diarrhea (Vallance et al., 2002)). While EPEC colonizes the small intestine, and EHEC the large intestine, both pathogens attach to intestinal epithelial cells and inject *E. coli* secreted proteins (Esps) into the host cell cytosol via the expression and functionalization of a type III secretion system (TTSS) (Abe and Nagano, 2000; Frankel et al., 1998). Esps A, B, and D are part of a translocon filament associated with the TTSS and are necessary for the translocation of effector molecules like Tir (translocated intimin receptor), EspF and MAP (mitochondria-associated protein) (Kenny et al., 2002; Roe et al., 2003). The Esps induce the reorganization and accumulation of the host cell cytoskeleton. This results in intimate attachment and effacement of the microvilli. The resulting characteristic pedestal-like formation beneath the attached bacterium is called the attaching and effacing (A/E) lesion (DeVinney et al., 2001; Goosney et al., 2001; Gruenheid et al., 2001; Nataro and Kaper, 1998) (Figures 1-1 and 1-2).

A/E lesion formation is dependant on Tir being delivered to the host cells and inserting into the host cell membrane where it acts as a receptor for the bacterial

Figure 1-1. Transmission Electron micrograph of a rabbit ileum thin section illustrating intimately adherent EHEC-related EPEC (Salyers and Whitt, 1994).

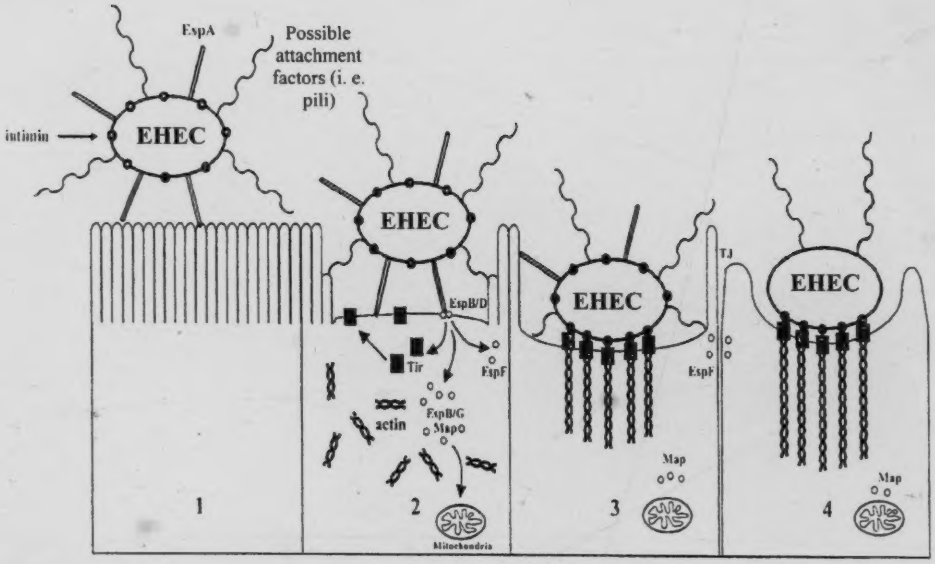
EspA
Possible attachment factors (i. e. pili)
intimin
EHEC
EHEC
EspB/D
Tir
EspF
actin
EspB/G
Map
Mitochondria
EHEC
EspF
TJ
Map
EHEC
Map
1
2
3
4

Figure 1-2. EHEC attachment, colonization and formation of A/E lesions. Step 1, Ingested EHEC comes in contact with intestinal epithelial cells of lower intestine, inducing the expression of Esps and possibly other attachment factors. Step 2, EHEC attaches intimately to intestinal epithelial cells via functionalization of TTSS and translocon filament, and the subsequent translocation of effector proteins like Tir, EspF and MAP proteins into the epithelial cell membrane. Step 3, The EspA translocon filaments are lost from the bacterial cell surface. The bacterial membrane protein, intimin, binds cellular membrane bound Tir resulting in intimate attachment. Translocated effector molecules induce host cell cytoskeleton rearrangement and formation of A/E lesions. Step 4, Accumulation of cytoskeletal components at the site of bacterial attachment induces pedestal formation. Tight junctions and mitochondrial function are impaired which eventually lead to death. This figure is adapted and modified from reviews published by S. C. Clarke and colleagues and A. J. Roe and colleagues (Clarke et al., 2003; Roe et al., 2003).

integral outer membrane protein, intimin (DeVinney et al., 2001; Kenny et al., 1997) (Figure 1-2). MAP has two functions; one at the mitochondria, where it disrupts mitochondrial membrane potential leading to apoptotic and necrotic cell death, and the other is independent of localization and induces filopodia formation (Clarke et al., 2003; Kenny et al., 2002). Filopodia are transient, independent of pedestal formation, and formed by cytoskeletal rearrangement. Filopodia form in the early stages of EHEC attachment and are down regulated once the interaction between Tir and intimin occurs. Until recently, filopodia were undetected and their function remains elusive. EspF is thought to disrupt tight junction integrity, and this activity contributes to inducing cell death (Crane et al., 2001).

The genes encoding the Esps and TTSS of EHEC are located on the chromosome in a pathogenicity-associated island called the locus of enterocyte effacement (LEE) (Figure 1-3) (Donnenberg and Whittam, 2001; Perna et al., 2001; Tarr et al., 2000).

Some investigators suggest the full adherence phenotype is dependant on the presence of a 90kb virulence-conferring plasmid pO157 (Karch et al., 1987; Tzipori et al., 1987). Mutagenesis and complementation experiments identified the pO157 *toxB* gene as a critical contributor to EHEC epithelial cell adherence, leading to the speculation that ToxB either promoted the production and/or secretion of type III secreted proteins (Figure 1-3) (Tatsuno et al., 2001). The virulence plasmid also encodes a hemolysin, EHEC Hly, that is present in the majority of EHEC serotypes, however, a clear role in pathogenesis has yet to be

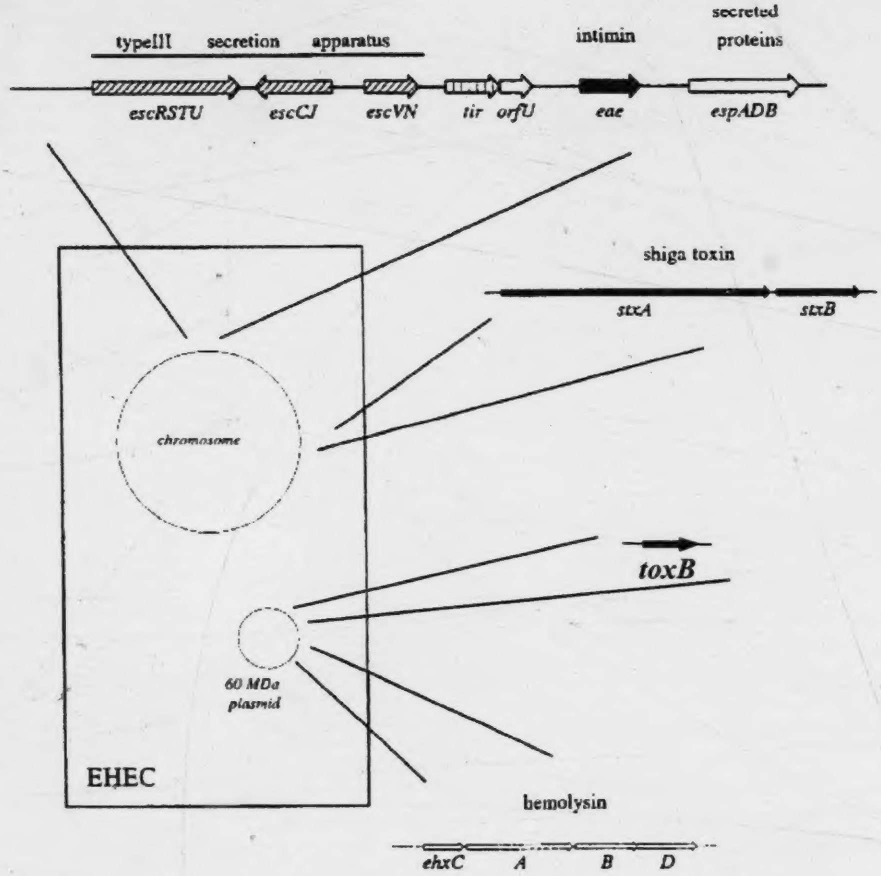
typeIII secretion apparatus
intimin
secreted proteins
escRSTU
escCJ
escVN
tir
orfU
eae
espADB
shiga toxin
stxA
stxB
chromosome
toxB
60 MDa plasmid
EHEC
hemolysin
ehxC
A
B
D

Figure 1-3. Genes involved in EHEC pathogenesis. LEE pathogenicity island (encodes TTSS, Esps, Tir and intimin) and the Stx-encoding phages are on the EHEC chromosome. The pO157 90kb plasmid (60-MDa plasmid) encodes the hemolysin EHEC Hly and the cellular adherence factor ToxB (Tatsuno et al., 2001). Adapted from a review article by J. P. Nataro and J. B. Kaper (Nataro and Kaper, 1998) and modified by the addition of the *toxB* gene encoded on the plasmid (Burland et al., 1998; Tatsuno et al., 2001).

established (Table 1-2 and Figure 1-3) (Meng et al., 1998; Schmidt et al., 1995).

Although colonization of the epithelial lining by EHEC leads to HC, it is not completely understood how this colonization process induces permeabilization of the infected intestinal epithelium, thereby allowing fluid to escape from the body (Cohen and Giannella, 1992). It is thought that the damage to the intestinal epithelium is augmented by the release of pro-inflammatory cytokines triggered by the colonization of the epithelial cells as well as release of Stx and lipopolysaccharide (LPS). (Cohen and Giannella, 1992; Dahan et al., 2002; Jacewicz et al., 1999; Thorpe et al., 1999; Thorpe et al., 2001).

1. 3. Stx

The AB_5-type Shiga toxins expressed by STEC are related to the Shiga toxin expressed by *Shigella dysenteriae* Type 1 (O'Brien and Holmes, 1987). STEC serotypes can express one or more structurally-related and functionally-related Shiga toxins; Stx1, Stx2, Stx2c, Stx2d and Stx2e, although only Stx1 and Stx2 are commonly associated with serotypes that infect humans (Ito et al., 1990; Lingwood, 1996). Stx1 and Stx2 share 56% amino acid identity and are immunologically distinct (Fraser et al., 1994; Jackson et al., 1987; Karmali et al., 1986; Scotland et al., 1985; Strockbine et al., 1986). Stx1 is a 71 kDa protein consisting of a 32 kDa A subunit and five identical 7.7 kDa B subunits that form a pentamer (Jackson et al., 1987) (Figure 1-4). Stx2 is slightly larger at 72 kDa, with a 33 kDa A subunit and 7.8kDa B subunits (Jackson et al., 1987). The Stx-

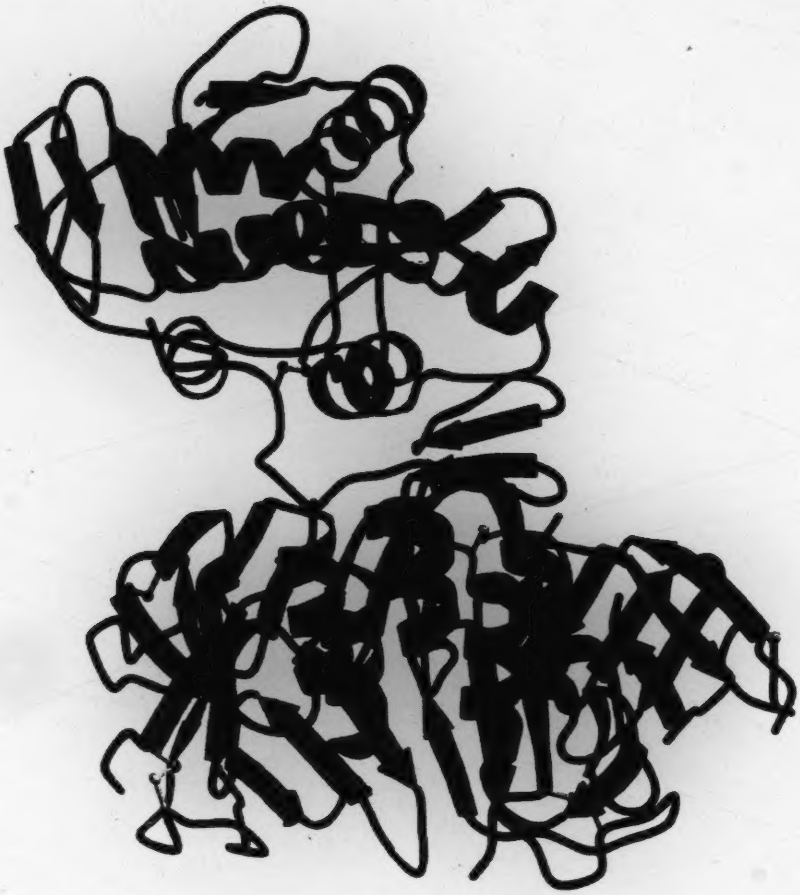

Figure 1-4. X-ray crystal structure of the Stx from *S. dysenteriae* at 2.5 angstrom resolution. The A subunit is depicted in red and the B subunits in green, purple, light blue and dark blue (Fraser et al., 1994).

encoding genes are located in a bacteriophage genome which has become integrated into the EHEC chromosome. The Stx bacteriophages can be transduced into other bacteria and therefore likely contribute to the emergence of new STEC variants (Schmidt, 2001).

1. 4. Stx and hemolytic-uremic syndrome

Once EHEC has colonized the gut it is possible for Stx to be released and spread from the intestines into the circulatory system where the toxins can then reach other target tissues and organs throughout the body. It is thought that the Shiga toxins traverse the gut and enter the circulation by a number of possible mechanisms (Acheson et al., 1996b; Hurley et al., 1999; Hurley et al., 2001). Low-level non-receptor mediated transcytosis of Stx has been shown to occur *in vitro* across polarized epithelial monolayers (Acheson et al., 1996b; Hurley et al., 1999). Alternatively, Stx may traverse the intestinal epithelium in association with polymorphonuclear cells (PMN's) (te Loo et al., 2000). PMN's are attracted to the infection site by pro-inflammatory cytokines which may also increase the permeability of the intestinal epithelial barrier and enhance Stx movement into the circulation (Hurley et al., 2001). PMN's, platelets and P-blood group antigens on the surface of human erythrocytes may bind Stx and the cells have also been proposed to act as Stx-carriers in the circulation (Bitzan et al., 1994; Karpman et al., 2001; te Loo et al., 2000) (Figure 1-5).

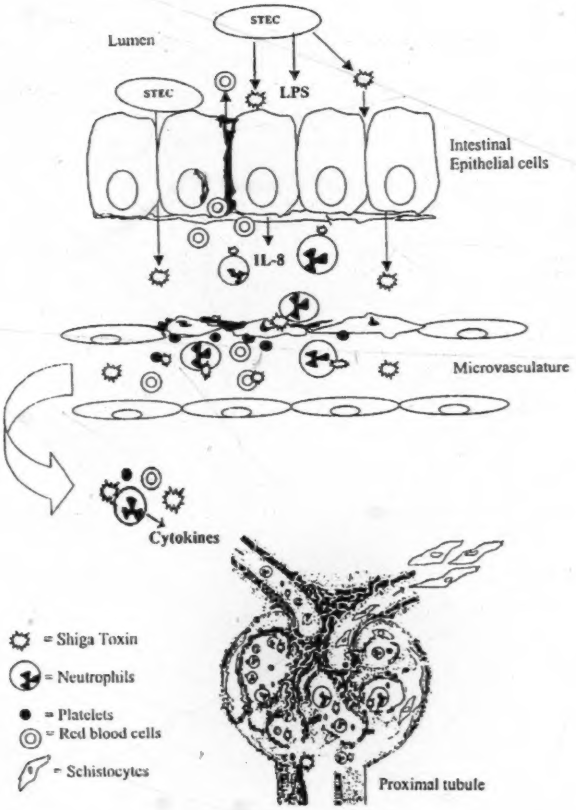
STEC
Lumen
STEC
LPS
Intestinal Epithelial cells
IL-8
Microvasculature
Cytokines
= Shiga Toxin
= Neutrophils
= Platelets
= Red blood cells
= Schistocytes
Proximal tubule

Figure 1-5. Possible pathways for Stx movement from the gut to renal target tissues. Intestinal Shiga toxin-producing *E. coli* (STEC) colonize the gut, produce Stx and induce the expression of pro-inflammatory cytokines. Stx can be translocated across the intestinal barrier via intra- or inter-epithelial pathways. Binding of Stx by red blood cells, platelettes and neutraphils induces the release of cytokines and facilitates Stx movement to target tissues (Ray and Liu, 2001).

The kidneys, and in some patients, the central nervous system are particularly susceptible to the toxic effects of circulating Stx. Stx also stimulates the release of pro-inflammatory cytokines such IL-6, IL-8 and TNF-α by neutrophils further augmenting the immune response and contributing to Stx-mediated toxicity (van Setten et al., 1996).

Many patients do not progress beyond hemolytic anemia, which is caused by Stx-induced microangiopathies and subsequent formation of microthrombi. However, in addition to hemolytic anemia, the Stx-mediated damage to glomerular endothelial cells can lead to renal thrombotic microangiopathies, thrombocytopenia and acute renal failure; the symptoms that characterize HUS (Louise and Obrig, 1994, 1995; Ray and Liu, 2001; Richardson et al., 1988). Stx-mediated damage of glomerular epithelial and tubular damage also appears to contribute to the development of HUS (Simon et al., 1998; Taguchi et al., 1998; Van Setten et al., 1997; Wadolkowski et al., 1990). Particularly in the elderly, Shigatoxemia may lead to a thrombotic thrombocytopenic purpura (TTP)-like syndrome, that has a high mortality rate (Carter et al., 1987; Ray and Liu, 2001). TTP is more disseminated than HUS, where organs other than the kidneys may be damaged. Neurological impairment may also occur in TTP.

1. 5. Stx and the cellular receptor

With the exception of Stx2e (pig edema toxin), the Shiga toxins specifically recognize a glycosphingolipid, globotriaosylceramide (Gb_3) found on many host cell surfaces (Boerlin et al., 1999; Lindberg et al., 1987; Waddell et al., 1988). Stx2e preferentially binds globotetraosylceramide (Gb_4). Gb_3 is a trisaccharide of galactose α (1-4) galactose β (1-4) glucosyl ceramide (Figure 1-6). The lipid moieties of Gb_3 are heterogeneous. The fatty acid chain length vary from 14 to 24 carbons and the degree of hydroxylation of the sphingosphine base is also variable. The presence and concentration of Gb_3 in the plasma membrane of a host cell is the primary determinant of sensitivity to Stx (Jacewicz et al., 1989; Lingwood, 1993, 1996; Waddell et al., 1990). In fact, it is thought that young children are at greater risk of developing HUS because they express greater levels of Gb_3 in glomerular endothelial cells (Lingwood, 1994). Cytokines and LPS can increase the expression of Gb_3 on host cell surfaces and thereby, increase cellular sensitivity to Stx (Hughes et al., 1998; van de Kar et al., 1992). However, Gb_3 expression does not preclude the possibility of resistance to Stx toxicity (Arab et al., 1998; Sandvig et al., 1994). Some Gb_3 expressing cells are completely insensitive to Stx and this has been proposed to be a result of differential intracellular trafficking which prevents the toxins from interacting with their cytoplasmic substrates (Arab and Lingwood, 1998). In Stx-insensitive Gb_3-expressing cell lines, the majority of the Stx is routed to lysosomes (Falguieres et al., 2001; Kiyokawa and Fujimoto, 2002).

This differential intracellular targeting is thought to be a function of the fatty acid composition of the Gb_3 and/or association with detergent resistant

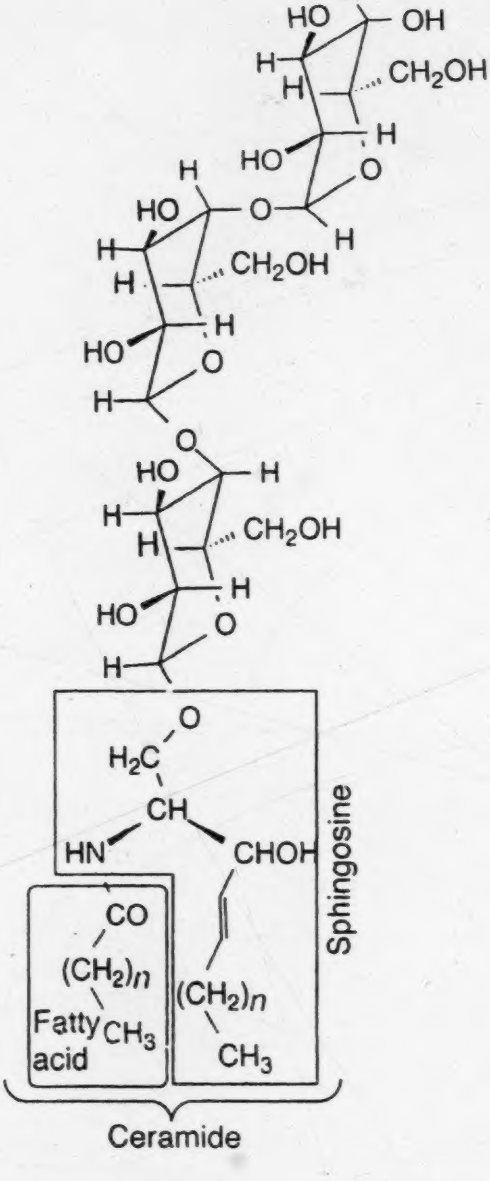
HO
H
OH
CH2OH
HO
O
CH2OH
CH3
(CH2)n
Fatty acid
CO
HN
CH
H2C
CHOH
Sphingosine
Ceramide

Figure 1-6. Structure of glycosphingolipid Gb_3 (Lingwood, 1996).

microdomains called lipid rafts (Falguieres et al., 2001; Hoey et al., 2003; Lingwood, 1996).

The B pentamer is the receptor binding component of the toxins. Studies of X-ray co-crystal structures combined with chemically synthesized analogue receptors has shown that each Stx1 B monomer possesses three possible carbohydrate receptor binding sites for a total of 15 binding sites per pentamer (Ling et al., 1998). These have been designated sites 1, 2 and 3. (Figure 1-7). Site-specific mutagenesis, in combination with solid-phase immobilization binding and cytotoxicity assays, confirmed the identity, importance and relevancy of all three binding sites *in vitro* (Bast et al., 1999). The multiple binding sites account for the relatively high affinity (K_d 4.6 x 10^{-8} M) of Stx1 for its Gb_3 receptors (Head et al., 1991). Stx2, by comparison to Stx1, has a lower affinity for its receptor with a K_d of 3.7 x 10^{-7} M (Head et al., 1991). Similar binding studies using Stx2e (87% identical to the Stx2 B subunit) mutated to recognize the Gb_3 instead of the Gb_4 receptor, show that the third site is not occupied in this toxin. This may indicate that Stx2 contains fewer functional binding sites than Stx1, but it is also possible that the third site is associated with residual Gb_4 binding in the Stx2e mutant (Ling et al., 2000).

1. 6. Stx internalization

The Stx B pentamer binding of Gb_3 on cell surfaces leads to toxin

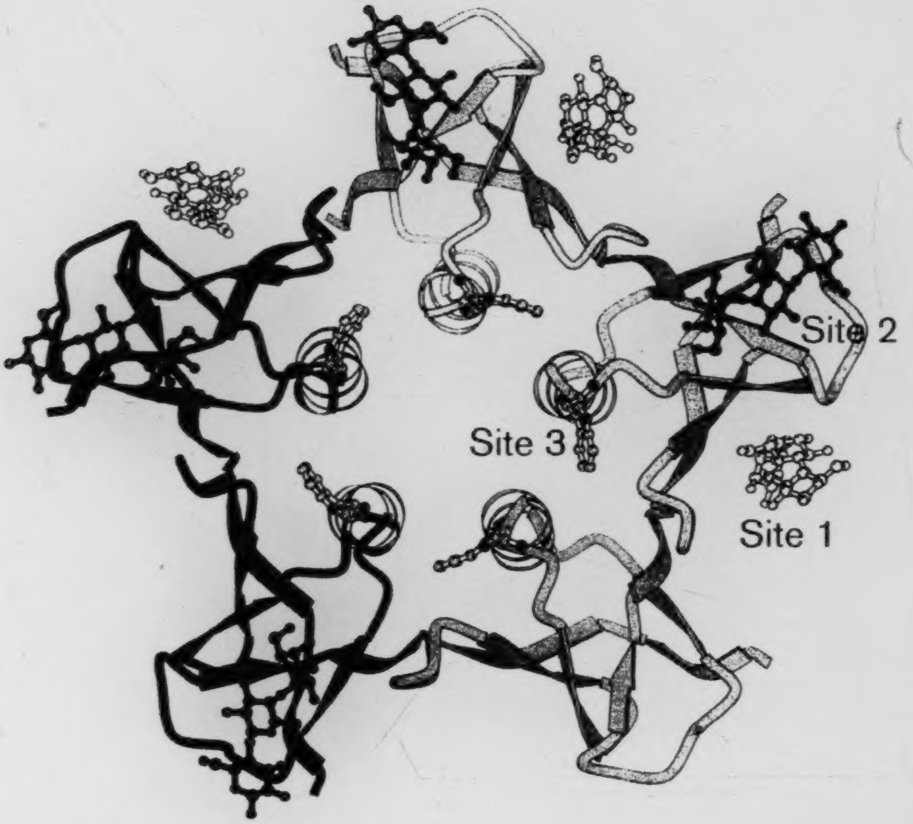

Figure 1-7. Co-crystal structure of the Stx1 B subunit complexed with an analogue of the Gb_3 receptor at 2.8 Angstrom resolution. The sugar is represented by the ball and stick motif. The figure represents the view along the five fold axis and shows the surface with the sugar binding domains facing the viewer (Ling et al., 1998).

internalization via endocytosis (Johannes, 2002; Khine and Lingwood, 1994; Sandvig et al., 2002; Sandvig and van Deurs, 2000, 2002). Stx1 can be detected in clathrin-coated pits (CCP) and clathrin-dependent endocytosis inhibitors antagonized Stx1-mediated cytotoxicity (Sandvig et al., 1989; Sandvig and van Deurs, 1996). Therefore, Stx internalization appears to occur via CCP despite ligating a glycolipid receptor that does not have a protein tail on the cytoplasmic inner leaflet side of the plasma membrane, which would typically be necessary for inducing signaling events that would cause aggregation of Stx to CCP. It is possible that by binding Gb_3, Stx may come in contact with an unidentified co-receptor that then initiates the signaling events required for receptor-mediated endocytosis. Stx may also be internalized by lipid rafts (caveolae), macro-pinocytosis, or other clathrin-independent mechanisms (Nichols et al., 2001; Sandvig and van Deurs, 1999, 2000). Subsequently, through retrograde transport, the Stx containing-endocytic vesicles move to the endoplasmic reticulum (ER) via the trans Golgi network (TGN) and finally to the cytoplasm (Sandvig et al., 1992). (Figure 1-8). How the toxin crosses into the cytoplasm is unknown but it may be utilizing an ER transport apparatus like the translocon utilizied by misfolded proteins (Swanton and Bulleid, 2003).

1. 7. Stx cytotoxicity

Once Stx is internalized, the RNA *N*-glycosidase activity of the A subunit on

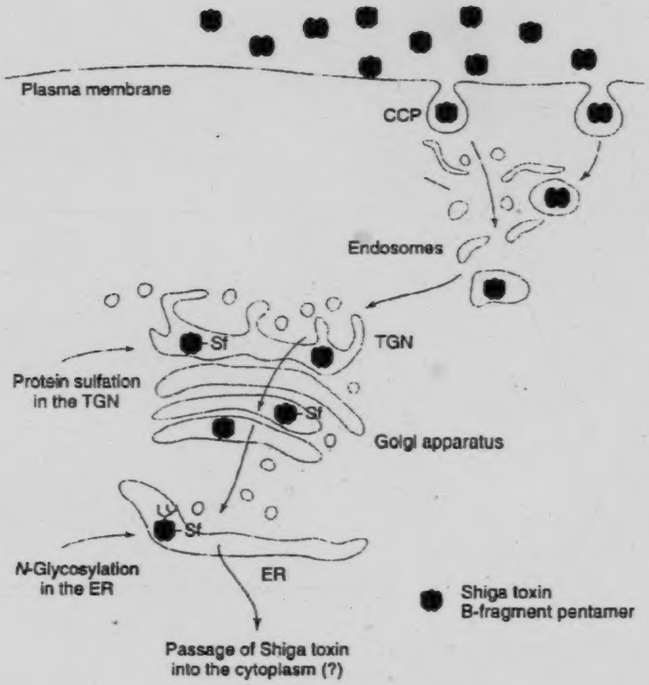
Plasma membrane
CCP
Endosomes
TGN
Sf
Protein sulfation in the TGN
Sf
Golgi apparatus
Sf
N-Glycosylation in the ER
ER
Shiga toxin B-fragment pentamer
Passage of Shiga toxin into the cytoplasm (?)

Figure 1-8. Internalization and retrograde transport of Stx via clathrin coated pits (CCP) or clathrin-independent pathways (Johannes and Goud, 1998).

host cell ribosomes is cytotoxic and triggers apoptosis (Kiyokawa et al., 2001; Kiyokawa et al., 1998; Mangeney et al., 1993; O'Brien et al., 1992). The A subunit removes an adenine from position 4324 of the 60S ribosomal subunit 28S rRNA component resulting in the arrest of protein biosynthesis by inhibition of peptide chain elongation (Endo et al., 1988; Reisbig et al., 1981). The A subunit disassociates from the B pentamer en route to the cytoplasm thereby freeing the A subunit catalytic activity (Reisbig et al., 1981; Sandvig and van Deurs, 2000).

The A subunit site of action at the ribosomal 28S rRNA is not specific to Stx as other highly potent toxins such as ricin and abrin have similar specificities (Fraser et al., 1994; O'Brien et al., 1992). Site-specific mutagenesis of the A subunit indicates that Glu 167 (Hovde et al., 1988), Arg 170 (Yamasaki et al., 1991) and Tyr 77 (Deresiewicz et al., 1992) probably occupy the N-glycosidase active site. The active site of the A subunit was confirmed by X-ray crystallography, where six active site residues were identified; Tyr 77, Tyr 114, Glu 167, Ala 168, Arg 170 and Trp 203 (Fraser et al., 1994).

In cell free protein synthesis assays, where the A subunit and B pentamers are not separated, the enzymatic activity of the A subunit can be freed by trypsin and dithiothreitol treatment (Reisbig et al., 1981). The A subunit is cleaved into a 27 kDa A1 fragment and 4 kDa A2 fragment, where the A1 fragment retains the hydrolytic N-glycosidase activity and specificity and the A2 fragment interacts with the B pentamer.

1. 8. Stx1 versus Stx2

While the expression of Stx1 or Stx2 by EHEC serotypes is implicated in human illness, epidemiological evidence indicates EHEC strains expressing both Stx1 and Stx2 or, Stx2 alone, are more likely to cause HUS than those expressing only Stx1 (Boerlin et al., 1999; Hashimoto et al., 1999; Kleanthous et al., 1990; Ostroff et al., 1989; Scotland et al., 1987). Stx2 is also more lethal than Stx1 in mice (Tesh et al., 1993; Wadolkowski et al., 1990) and baboons (Siegler et al., 2003) and causes greater intestinal pathology in rabbits (Head et al., 1988). Although the exact mechanism for the different *in vivo* toxicities remains unclear, it may be related to a combination of factors, which are discussed below.

The degree of Stx1 and Stx2 cytotoxicity is cell-dependant with neither toxin being uniformly more cytotoxic. Stx1 is 10 fold more cytotoxic than Stx2 in Hela and Vero cells (Acheson, 1993), and equally cytotoxic in other cell lines (Mangeney et al., 1993; Ohmi et al., 1998). Stx2 is more cytotoxic to intestinal microvascular endothelial cells (Jacewicz et al., 1999) and up to 1000 fold more cytotoxic than Stx1 in glomeruli endothelial cells (Louise and Obrig, 1995). As discussed in an earlier section (1. 5.), Stx1 has a higher affinity for its receptor, and there tends to be a direct relationship between the amount of receptor expression and Stx cell sensitivity. Therefore, following this reasoning, Stx1 should be more cytotoxic than Stx2. In one study when the A and B subunits of Stx1 and Stx2 were dissociated and separated using urea, proprionic acid and High Performance Liquid Chromatography (HPLC) gel filtration, and then re-assembled into hybrid toxins, the *in vitro* cytotoxicity phenotype was dependant on the source of the B subunit (Head et al., 1991). Similarly, in an *in vivo* rabbit toxicity study using the hybrid toxins, it was again demonstrated that the B subunit was the major determinant of the cytotoxic phenotype (Nelson et al., 1994).

At super-physiological concentrations the Stx1 B subunit induces apoptosis (Mangeney et al., 1993), leading to the hypothesis that, in addition to A subunit-mediated inhibition of protein biosynthesis, the Shiga toxins may employ additional cytotoxic mechanism(s). Ligation of Gb_3 by the B pentamer could possibly induce cell signaling events that would directly or indirectly initiate the caspase-cascade and lead to apoptosis (Mangeney et al., 1993; Taga et al., 1997). This has led some to speculate that the Stx B subunits may play an unrecognized role in EHEC pathogenesis (Jones et al., 2000; Mangeney et al., 1993; Nakagawa et al., 1999; Pijpers et al., 2001).

The different *in vivo* toxicities of Stx1 and Stx2 has also been proposed to be due to the differential tissue localization and serum half lives of Stx1 and Stx2. N. Rutjes and colleagues found that Stx2 remains in circulation longer than Stx1 and Stx1 rapidly localizes to the nasal turbinate region and lungs of mice while Stx2 favors greater renal targeting in mice (Rutjes et al., 2002).

1. 9. Stx and antibodies

Studies on the prevalence of Stx neutralizing antibodies in EHEC-infected and non-infected populations have produced some notable findings. M. Bitzan and colleagues (Bitzan et al., 1993b) found that 9% of HUS patients in Germany produced neutralizing antibodies to Stx1, and H. Barrett and colleagues (Barrett et al., 1991) found a frequency of 20% of HUS patients with Stx1-specific antibodies in the United States (Barrett et al., 1991; Bitzan et al., 1993b). This is in contrast to control populations where 2.5% of study subjects in Germany, and 10.6% of study subjects in the United States showed evidence of an anti-Stx response (Bitzan et al., 1993b) (Barrett et al., 1991). Results with Stx2 have been more confounding, as several investigators showed that not only do all the serum samples of HUS patients neutralize Stx2, but all serum samples of control patients yielded similar neutralization results (Bitzan et al., 1993a; Bitzan et al., 1993b; Caprioli et al., 1994a). The ubiquitous Stx2-neutralizing factor is present in the immunoglobulin-depleted fractions of these serum samples (Bitzan et al., 1993a). Notably, this non-immunoglobin factor was only present in humans. Bovine, pig, mouse, rat, hamster, guinea pig, rabbit, chicken, dog, sheep, deer, goat, horse, and monkey sera were all tested and were negative for this non-immunoglobulin Stx2 neutralizing factor (Caprioli et al., 1994a; Kimura et al., 2001). The identity of the Stx2-specific serum factor was finally discovered to be human serum amyloid P component (HuSAP) (Kimura et al., 2001; Marcato et al., 2003b).

1. 10. Human serum amyloid P component (HuSAP)

Like the classical acute phase protein, C-reactive protein (CRP), SAP is a pentraxin plasma protein and is part of the innate immune system; the body's first line of defense against pathogens. (Emsley et al., 1994; Pepys et al., 1997). In adult humans, HuSAP concentrations in the circulation range from 30 to 45 μg/mL and can double during infections (Nelson et al., 1991; Pepys et al., 1982). These proteins belong to a highly conserved lectin fold super family (Emsley et al., 1994; Thompson et al., 1999). SAP is a pentameric protein consisting of five identical non-covalently associated monomers arranged in cyclic five-fold rotational symmetry, much like the B pentamer of Stx. SAP is a 125 kDa protein with each monomer having a molecular weight of 25 kDa. Two SAP pentamers can homopolymerize in a "face-to-face" orientation in the presence of calcium and specific ligands (Emsley et al., 1994).

SAP is known to bind amyloid fibrils, resulting in the ubiquitous presence of SAP in amyloid deposits. This contributes to the stabilization of amyloid fibrils and pathogenesis of amyloidosis conditions (Botto et al., 1997; Pepys, 2001; Tennent et al., 1995). However, the natural function of SAP appears to be the binding and clearance of pathogens or host-derived cellular debris. SAP binds lipopolysaccharide (LPS) and Gram negative bacteria (de Haas, 1999). SAP also binds to extracellular DNA in chromatin, released by apoptosis or necrosis, stabilizing it and preventing its degradation (Bickerstaff et al., 1999; Breathnach et al., 1989; Pepys and Butler, 1987). M. C. Bickerstaff and colleagues proposed that the binding of SAP to chromatin prevents autoimmunity to cellular DNA (Bickerstaff et al., 1999).

1. 11. HuSAP interaction with Stx2

T. Kimura and colleagues, who first described the association of Stx2 with HuSAP, suggested that this specific interaction may be relevant to EHEC pathogenicity (Kimura et al., 2001). These investigators also showed that Stx1 did not interact with HuSAP (Kimura et al., 2001) and postulated that the binding of Stx2 to HuSAP may also contribute to the enhanced *in vivo* toxicity of Stx2 by acting as a carrier and facilitating the toxin transport to target organs.

1. 12. EHEC and animal models

A variety of animal models can be used for studying EHEC pathogenesis. Mice, rabbits and pigs represent established and acceptable models for studying Shigatoxemia, and support the theory that organs expressing the greatest density of the Stx receptor are the most susceptible to tissue damage. These models also demonstrate that the Shiga toxins play a critical role in EHEC pathogenesis (Dykstra et al., 1993; Fujii et al., 1996; Rutjes et al., 2002; Tesh et al., 1993). Purified Stx can be administered intravenously, intraperitoneally, intramuscularly or subcutaneously with similar organ targeting and lethal effects. Without further manipulation, however, the glomerular endothelial cells in these animals do not express Gb_3 to the same extent as humans and therefore renal thrombotic microangiopathies that are the hallmark of HUS in humans do not develop. Generally, histopathology indicates a more systemic toxic effect with stronger evidence of neuropathology.

EHEC colonization models have been developed in attempts to mimic the conditions of the human infection where Stx must be secreted by the bacteria in the gut, traverse of the intestinal epithelial barrier and then enter the circulation. In the mouse infection model, either gnotobiotic mice (Isogai et al., 1998; Lai et al., 1991; Nishikawa et al., 2002), or mice treated with antibiotics to disrupt the indigenous microflora can be used (Fujii et al., 1994; Lindgren et al., 1993; Wadolkowski et al., 1990). In these models a high dose must to be administered (10^7 Cfu) and although the mice display the toxic effects of Stx2, they do not develop diarrhea. The infant rabbit model possibly represents a better EHEC infection model with the advantages of reproducible diarrhea, a lower infectious dose of 10^3 Cfu, colonization of the lower intestine and susceptibility to Stx toxicity (Ogawa et al., 2001; Pai et al., 1986). Gnotobiotic piglets orally infected with Stx2 producing EHEC developed both intestinal and extra-intestinal disease pathology, including thrombotic microangiopathies (Gunzer et al., 2002).

A recognized superior model of infection is the greyhound dog, originally identified after it was discovered that the "Alabama Rot" these animals suffered was due to an EHEC infection contracted from the consumption of contaminated meat (Hertzke et al., 1995). These animals develop bloody diarrhea, acute renal failure and renal glomerular lesions that resemble the thrombotic microangiopathic lesions seen in human HUS cases. Conversely, the dogs also develop skin ulcers and edema that is not seen in humans. Baboons injected with Stx, particularly Stx2, develop thrombotic microangiopathies and HUS, however the expense and facilities required for working with these animals make experimenting with them difficult (Siegler et al., 2003; Taylor et al., 1999).

1. 13. EHEC infections and antibiotics

Antibiotics, the first line of defense against bacterial infections, are generally not recommended as a treatment for EHEC-infected individuals (Zimmerhackl, 2000). Several studies have shown that antibiotic treatment does improve the outcome for the patient (Bell et al., 1997; Ryan et al., 1986). Others have correlated the use of antibiotics with a significant increased risk of developing HUS and mortality (Carter et al., 1987; Pavia et al., 1990; Wong et al., 2000). These results contrast with the findings of a Japanese outbreak study where fosfomycin treatment administered on or before the second day of illness was associated with a significant decrease in HUS (Ikeda et al., 1999). Therefore, the use of antibiotics in treating EHEC infections remains controversial and the focus of continuing studies. Much of the controversy stems from the time at which antibiotic treatment was begun as most often it was the more severely ill children that received antibiotic therapy in these studies. Therefore, these subjects may have already been well along the road to developing HUS, confounding the association between the risk of developing HUS and antibiotic treatment (Wong et al., 2000).

In vitro experiments show that exposure to some antibiotics causes increased release of Stx from the periplasm by EHEC (Grif et al., 1998; Ito et al., 1997; Karch et al., 1986; Kimmitt et al., 1999; Mulvey et al., 2002). Therefore, it is possible that antibiotics may increase the risk of HUS by promoting the release into the gut of Stx, which can then enter the systemic circulation and reach target tissues.

Currently, there are no other effective treatments approved for human use. Therefore, developing novel therapeutics for EHEC or preventing EHEC infections by vaccination are worthwhile pursuits.

1. 14. Novel therapeutics

It was proposed that Synsorb-Pk, an indigestible material derivatized with synthetic Stx Gb_3 (Pk is a minor blood group antigen) receptor sequences, could be used to absorb the toxins in the gut and aid in their elimination before they caused HUS and HC in EHEC-infected subjects. This theory was based on *in vitro* data where co-incubation with Synsorb-Pk prevented Stx-mediated cytotoxicity. The cumulative data from Phase II clinical trials suggested there was a non-significant trend in favor of the efficacy of Synsorb Pk in decreasing the risk of developing HUS (Armstrong et al., 1998). The inventors proposed that the failure of Synsorb-Pk to significantly ameliorate the outcome in EHEC-infected patients was due to its retention within the intestinal lumen (Mulvey et al., 2003). As discussed in section 1. 4., Stx enters the circulation via inter- and intra-intestinal epithelial mechanisms (Acheson et al., 1996b; Hurley et al., 1999; Hurley et al., 2001). Therefore, once Stx leaves the intestines, it is lost to the therapeutic actions of the indigestible Synsorb-Pk.

Based on these arguments, P. I. Kitov and colleagues (Kitov et al., 2000) utilized X-ray crystallographic information (Ling et al., 1998) to rationally design a high affinity tailored, water-soluble carbohydrate Stx inhibitor (Figure 1-9). Nicknamed "Starfish", the pentameric multivalent inhibitor combined five pairs of Pk-trisaccharides tethered to a central core (Kitov et al., 2000). Sub-micromolar concentrations of Starfish inhibited Stx-mediated cytotoxicity *in vitro*. A modified version of Starfish, called "Daisy", was used to protect mice challenged with lethal doses of purified Stx1 or Stx2 and also in an STEC infection model (Figure 1-10) (Mulvey et al., 2003). Others have employed similar strategies for developing EHEC treatments. Another water soluble multivalent Stx inhibitor, SUPER TWIG, is one of a series of carbosilane dendrimers carrying six Pk-trisaccharides that effectively neutralized lethal doses of Stx2 in an EHEC gnotobiotic mouse infection model (Nishikawa et al., 2002).

An alternative to the Synsorb-Pk ingestible Stx inhibitor, is a genetically modified *E. coli* that displays LPS that mimics the Stx receptor (Paton et al., 2000). The modified *E. coli* binds Stx with increased affinity over Synsorb-Pk,

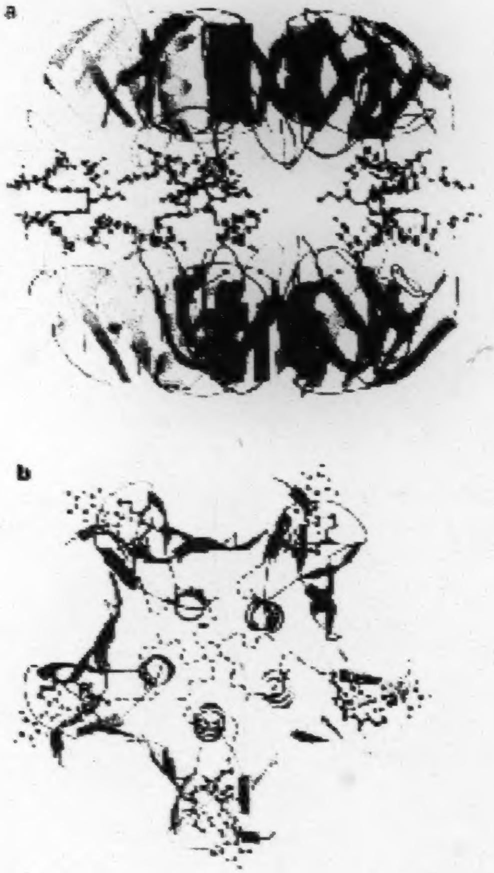

Figure 1-9. Mode of binding in the Stx1-Starfish complex. Diagram of the crystallographic dimer of Stx1 B-subunit pentamers linked by the Starfish ligand (a). The toxin β-strands are illustrated as broad arrows and α-helices as coiled ribbons. Starfish is shown in a ball-and-stick representation. Diagram of half of the Starfish-Stx1 B sandwich (b). The representation is as in (a), except that dashed magenta lines show a possible conformation for the central component of the linker, which could not be seen clearly in the electron density (Kitov et al., 2000).

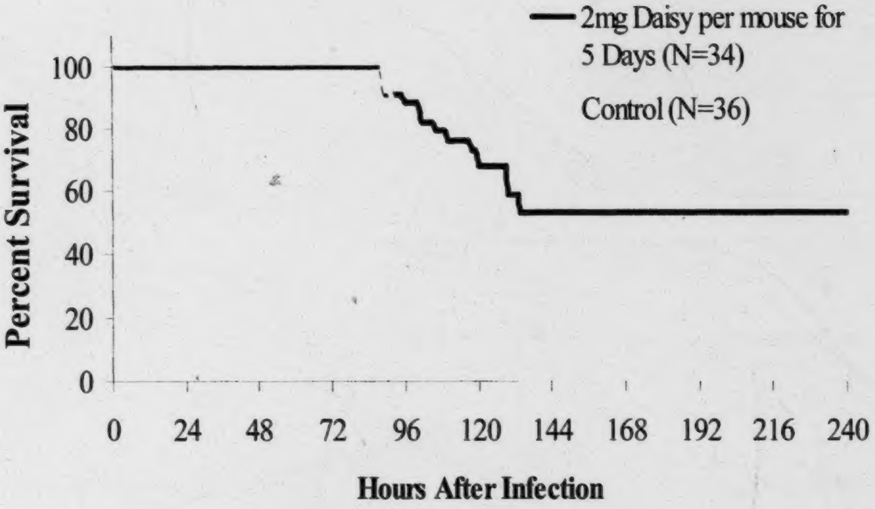

Figure 1-10. Survival plot demonstrating the protective effect of Daisy in the *Escherichia coli* O91:H21 infection challenge model. After 24 h, the mice each received a single anterior dorsal injection of either Daisy in PBS–Quil-A or PBS–Quil-A alone. These injections were repeated on a daily basis until all the animals in one of the groups were euthanized when obvious signs of Shigatoxemia became apparent (Mulvey et al., 2003).

and ingestion of the bacteria protected EHEC-infected mice from the lethal effects of the toxins.

Monoclonal antibodies to both the A and B subunits of Stx have also been proposed as therapeutic agents (Mukherjee et al., 2002; Sheoran et al., 2003; Yamagami et al., 2001). The antibody treatments were effective at increasing survival in mouse toxicity and infection models and in a pig infection model.

All these treatments show promise and could ameliorate the disease outcome for an EHEC-infected patient where the risk of developing HUS is reduced. Unfortunately, all these treatments suffer from the limitation that they fail to provide any benefit if treatment is not initiated very soon after the toxin is released into the systemic circulation. For example, Daisy, (Mulvey et al., 2001) or TMA-15, the therapeutic Stx monoclonal antibody (Yamagami et al., 2001), were only effective when given 24 hours after the *E. coli* infection was initiated. A time-course analysis of the mouse serum Stx2 levels detected toxin only 24 h after infection (Yamagami et al., 2001). Therefore, based on these animal studies, unless the treatment is commenced as soon as the toxin is present in the system, the drug is rendered ineffective.

However, the illness in *E. coli*-challenged mice is also significantly different from that occurring in humans. In humans, EHEC infections resulting in death is rare. By contrast, in the mouse model the EHEC infection is 100% lethal. Also, mice used in these experiments do not develop obvious intestinal signs of infection and are without symptomology for at least 4 days post infection.

Symptoms of Shigatoxemia can often occur only 2 to 4 hours prior to death in the mice. Therefore, it is possible that the limited therapeutic window for the drugs in mice does not reflect the situation in humans in which the disease dynamics and clinical outcomes could be drastically different.

There is limited data suggesting that the therapeutic window is narrow in humans. For instance preliminary data from the Synsorb-Pk clinical trial suggests a 54% reduction in the risk of developing HUS, but only if drug delivery was commenced within 3 days of the onset of diarrhea (Armstrong et al., 1998). Similarly, if fosfomycin was administered to patients on or after the third day of illness, then the treatment was rendered ineffective at preventing HUS (Ikeda et al., 1999). Therefore, unless these novel therapeutics can be effective in a broader treatment window, it is unlikely they would be useful drugs in treating EHEC infections and HUS (Ikeda et al., 1999). In addition, carbohydrate drugs like Starfish and Daisy require complex chemical synthetic processes, making the cost of manufacture expensive and perhaps prohibitive.

1. 15. EHEC cattle vaccine

Y. Li and colleagues assayed the sera of EHEC-infected patients for specific antibodies against several of the virulence factors involved in colonization and formation of A/E lesions (Li et al., 2000). Using this methodology, the authors identified four possible EHEC virulence factors that could possibly be incorporated into an EHEC vaccine; EspA, EspB, Tir and intimin (Li et al., 2000).

Further testing as to the feasibility of using these purified proteins as human immunoprophylactic agents has not been pursued. Instead, these proteins involved in EHEC attachment and colonization have been advocated as components in a cattle vaccine (Vallance et al., 2002).

While using purified recombinant proteins to vaccinate humans is acceptable, in cattle such a vaccine mixture would be overly expensive and therefore impractical. To overcome the prohibitive cost, the inventors propose to use the cell culture supernatant (CCS) of O157 *E. coli* under conditions where these TTSS proteins (EspA and Tir) are produced in quantity (Finlay and Potter, 2002). The theory is that cattle immunized with O157:H7 CCS would produce an immune response against these attachment-mediating proteins, thereby inhibiting colonization of the bovine gut and reducing shedding of the organisms in their feces (Figure 1-11). Theoretically, if the source of EHEC contamination is reduced, then the chance of humans coming into contact with the organisms will be diminished. The effectiveness of the O157 cattle vaccine is currently being tested along side a commercially available *Lactobacillus acidophilus* feed additive in 1100 cattle at the University of Nebraska. The Calgary Herald recently reported that another feedlot trial by the Alberta Research Council (ARC) employing 30000 cattle that began in 2001 was halted after the results were deemed inconclusive due to flawed protocols (2003). The ARC will resume the trials by the end of 2004, with smaller groups of animals that will be individually tested to improve the reliability of the results. Therefore, it appears that

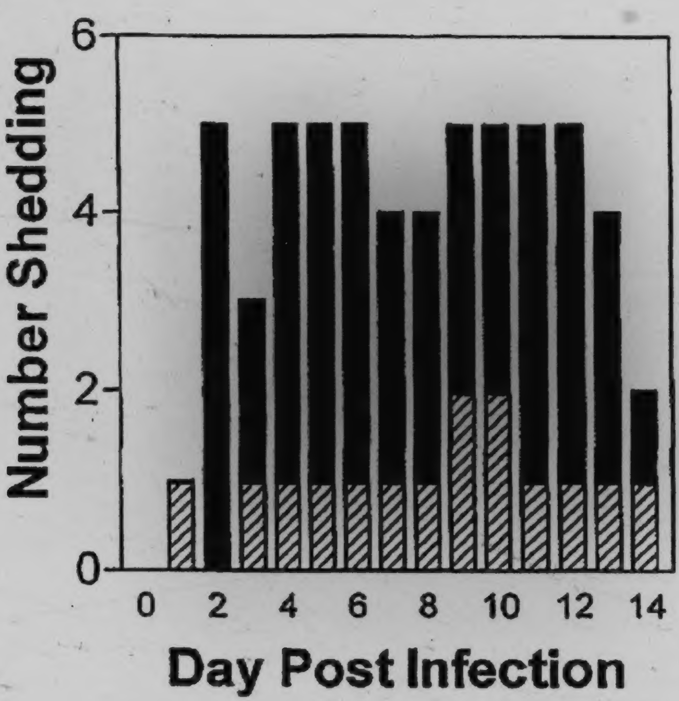

Figure 1-11. The number of cows shedding *E. coli* O157:H7 on each day post vaccination with CCS (hatched bars) or saline (black bars). The animals (n=8) were immunized on days 1 and 22 and on day 36 were challenged with 10^8 CFU of EHEC (Finlay and Potter, 2002).

The total number of shed EHEC isolated from fecal samples was significantly lower (Wilcoxin Signed Rank test, p = 0.05) with the CCS immunized animals shedding a median value of 6.25 CFU/g of feces and the saline immunized animals shedding a median value of 81.25 CFU/g of feces (Finlay and Potter, 2002).

determining the effectiveness of the bovine EHEC vaccine at reducing shedding of the organism into the environment has not been trivial and it remains uncertain if this approach will ever be feasible. Even if the vaccine does eventually prove effective, unless the reduction in shedding is sufficient, the end result of reducing the number of EHEC infections in humans may not be significant. It should also be considered, that while cattle represent a major source of EHEC production in the environment, they are not the only source, as other ruminants such as sheep also shed EHEC (Rocchi and Capozzi, 1999). Nevertheless, this bovine vaccine could also be used to immunize these other EHEC carriers (Finlay and Potter, 2002).

1. 16. EHEC human vaccines

Several approaches have been proposed for an EHEC vaccine in humans. Live attenuated or formaldehyde-killed whole cell lysate vaccines against some EHEC serotypes O157:H7, O26:H11, O139:H1 have been proposed (Tana et al., 2003; Yamasaki, 2002). While there is no denying the effectiveness of such vaccines at inducing an immune response, modern vaccines approved for human use are typically acellular and based on highly purified bacterial antigens that are less likely to induce toxic side effects. O-specific polysaccharide conjugate vaccines have also been proposed that are designed to induce an anti-LPS immune response that will inactivate the EHEC inoculum and prevent colonization (Conlan et al., 2000; Konadu et al., 1999; Konadu et al., 1994; Konadu et al., 1998). Alternatively, immunization with live Salmonella that express the O antigens of EHEC serotypes O157:H7 or 0111 have also been proposed (Conlan et al., 1999; Wang et al., 1999). While these approaches have achieved some success at inducing an immune response, these serotype-specific vaccine formulations suffer from the inevitability that EHEC infections are caused by a large number of serotypes and therefore, are not ideally suited to serve as a universal EHEC vaccine.

The immunoprophylactic potential of the Shiga toxins has also been studied *in vivo*. The universal expression of Stx1 and/or Stx2 by virtually all EHEC serotypes that cause human infection, in addition to their important role in the life-threatening complications in such infections, makes these antigens attractive acellular vaccine components. Rabbits or mice immunized with the recombinant Stx1 B subunit developed neutralizing antibodies to the toxin and were protected against a subsequent Stx1 holotoxin challenge (Acheson et al., 1996a; Boyd et al., 1991; Butterton et al., 1997; Byun et al., 2001). Given the aforementioned importance of Stx2 in EHEC disease, the need for a protective Stx2 immune response in any potential Stx-based EHEC vaccine would be necessary. *In vitro* assays indicate that Stx1 and Stx2 are serologically distinct and antibodies to these toxins do not cross-neutralize their activities (Karmali et al., 1986; Scotland et al., 1985; Strockbine et al., 1986). In contrast, numerous studies in animals immunized with Stx1 or Stx2 toxoid preparations indicate that animals are cross-protected against a subsequent challenge by either toxin (Bielaszewska et al., 1997; Bielaszewska et al., 1994). This cross-protection was primarily due to the production of A subunit-specific antibodies. Additional studies demonstrated that antibodies to the Stx1 or Stx2 B subunits are not cross-protective (Bielaszewska et al., 1997; Bielaszewska et al., 1994; Wadolkowski et al., 1990).

1. 17. Aims of Thesis

At the outset of my studies, I postulated that the recombinant Stx2 B subunit might represent a possible candidate for inclusion in an acellular human EHEC vaccine. I also postulated that the recombinant Stx2 B subunit could be used as a tool in studies designed to increase our understanding of EHEC pathogenic mechanisms. The goal of my thesis work, therefore, was to clone and express the Stx2 B subunit such that it could assemble into stable pentamers that recognized Gb_3 receptors. Once cloned, the Stx2 B subunit could be tested for its immunoprophylactic potential in rabbits. Alternatively, mice could be used in the immunoprophylactic studies with the Stx2 B subunit as their small size is advantageous when large numbers of animals are required. The recombinant Stx2 B subunit could also be tested for possible A subunit-independent apoptogenic activity which, for safety reasons, might preclude its use without further modification in a human vaccine. Since HuSAP is thought to have immunomodulatory properties (Bickerstaff et al., 1999; Breathnach et al., 1989; Pepys and Butler, 1987), HuSAP binding to Stx2 could possibly affect the Stx2 immune response. Therefore, the recombinant Stx2 B subunit was also used to further characterize the interaction between Stx2 holotoxin and HuSAP, and determine whether this interaction was specific to the A subunit or B pentamer. The results of these investigations are described in the following chapters of my thesis.

Chapter 2

Immunoprophylactic Potential of Cloned Shiga Toxin 2 B Subunit

A version of this chapter has been published. Marcato, P., Mulvey, G., Read, R. J., Vander Helm, K., Nation, P. N., Armstrong, G. D., Journal of Infectious Diseases, volume 183, p. 435-443, 2001. Copyright 2001. The University of Chicago press.

G. Mulvey contributed intellectually and helped monitor and euthanize animals in the challenge experiment. K. Vander Helm helped monitor and euthanize animals in the challenge experiment. Dr. R. J. Read contributed intellectually. Dr. P. N. Nation performed necropsies on the euthanized animals.

This work was supported by a grant to GDA from the Canadian Bacterial Diseases Network.

Figures 2-3, 2-4 and 2-6 appeared as "data not shown" in the Journal of Infectious Diseases version of this thesis chapter.

In this chapter, I describe how I overcame the difficulties encountered in cloning and expressing the Stx2 B subunit. I then examined the immunoprophylactic potential of the resulting antigen in the rabbit Stx2 challenge model.

2. 1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) cause hemorrhagic colitis (HC) and, on occasion, a life-threatening complication, hemolytic-uremic syndrome (HUS) in humans (Gianviti et al., 1994; Karmali et al., 1986; Sjogren et al., 1994; Takeda, 1993). EHEC are also known as Shiga toxin-producing *E. coli* (STEC) or Verotoxin-producing *E. coli* (VTEC) (Karmali, 1989). Six different Shiga toxins (Stx) have been described, including the one from *Shigella dysenteriae*, the closely related *E. coli* Stx1, and the more distantly related *E. coli*, Stx2, Stx2c, Stx2d, and Stx2e. The *E. coli* Stx1 and Stx2 are associated with STEC serotypes involved in human infections and are linked to the development of HC and HUS (Lingwood, 1996).

Although all six varieties of Stx differ to some degree at the amino acid sequence level (Jackson et al., 1987), these toxins are all multimeric proteins which display a classic AB_5 structure (Fraser et al., 1994; Stein et al., 1992). The Stx B subunits form a toroid-shaped pentamer that, with the exception of the Stx2e B subunit, recognize globotriaosylceramide (Gb_3) receptors found on a number of different target cells (Jacewicz et al., 1986; Lindberg et al., 1987; Waddell et al., 1988). Upon receptor ligation, the host cell internalizes the toxin, the A-B subunits dissociate, and the A subunit's *N*-glycosidase activity then removes adenine from position 4328 of the eukaryotic 28s rRNA (Endo et al., 1988; Obrig et al., 1987; Reisbig et al., 1981). The resulting A subunit-mediated inhibition of protein biosynthesis is cytotoxic to the target cell.

Many different STEC serotypes have been isolated from human cases of HC and HUS. In North America, O157:H7 remains the dominant serotype, while in other regions of the world, serotypes such as O145:H-, O111:H-, Out:H19 prevail (Caprioli et al., 1994b; Takeda et al., 1997). Regardless of serotype, Stx1 and/or Stx2 are uniformly expressed by virtually all STEC from human cases. This universal expression of these toxins, in addition to their important role in the life-threatening complications of STEC infections, makes these antigens attractive immunoprophylactic targets (Mulvey et al., 1998)

In vitro assays indicate that Stx1 and Stx2 are serologically distinct and antibodies to these toxins do not cross-neutralize their activities (Karmali et al., 1986; Scotland et al., 1985; Strockbine et al., 1986). In contrast, numerous studies in animals immunized with Stx1 or Stx2 toxoid preparations indicate that animals are cross-protected against subsequent challenge by either toxin (Bielaszewska et al., 1997; Bielaszewska et al., 1994). However, this cross-protection was primarily due to the production of A subunit-specific antibodies. Additional studies demonstrated that antibodies to the Stx1 or Stx2 B subunits are not cross-protective (Bielaszewska et al., 1997; Bielaszewska et al., 1994; Wadolkowski et al., 1990).

Published evidence indicates a closer association between Stx2 expression by STEC and a more severe course of illness (Hashimoto et al., 1999; Ostroff et al., 1989; Scotland et al., 1987). For example, in a recent outbreak involving 131 Japanese patients infected with an Stx1-producing O118:H2 STEC serotype, not one case of HUS was reported and the gastrointestinal symptoms in these individuals were relatively mild (Hashimoto et al., 1999). This contrasts with other documented STEC outbreaks, involving serotypes producing a combination of Stx1 and Stx2, or Stx2 alone, in which the incidence of HUS ranged between 5% to 10% of infected subjects (Karmali et al., 1985; Neill et al., 1987; Slutsker et al., 1998). In addition, a study of sporadic HUS cases, indicated that 94% involved STEC serotypes capable of producing Stx2 alone or a combination of Stx2 and Stx1, while only 6% of cases developed HUS after infection with an STEC serotype that produced Stx1 only (Kleanthous et al., 1990). Finally, Stx2 is more lethal than Stx1 in mice (Tesh et al., 1993), and causes a greater amount of intestinal pathology in rabbits (Head et al., 1988).

The immunoprophylactic potential of the Stx1 B subunit has been thoroughly documented in the literature (Acheson et al., 1996a; Boyd et al., 1991; Butterton et al., 1997). However, given the importance of Stx2 in STEC disease, and that anti-Stx1 B subunit antibodies do not confer protection against a Stx2 holotoxin challenge, any potential STEC vaccine must generate a protective Stx2 immune response. Although animals immunized with Stx2 toxoid preparations are protected against a holotoxin challenge (Bielaszewska et al., 1997); there are safety concerns associated with using inactivated holotoxins in human vaccines, especially when the incidence of the disease is low. Therefore, if a practical Stx-based acellular STEC vaccine is to be developed, the atoxic Stx2 B subunit would be a potential component.

Unlike the cloning and expression of the Stx1 B subunit (Acheson et al., 1993; Calderwood et al., 1990; Ramotar et al., 1990), cloning and expression of the Stx2 B subunit has proved to be more challenging (Acheson et al., 1995). It has been suggested that this might be due to the instability of the multimeric Stx2 B subunit in *E. coli* expression systems (Acheson et al., 1995). We therefore speculated that the difference between the expression of the Stx1 B and Stx2 B subunits might be in the processing steps involved in their secretion into the periplasmic space in *E. coli*. Following on this reasoning, we discovered that interchanging the Stx1 and Stx2 B leader peptide nucleotide coding sequences solved the Stx2 B cloning problem. We subsequently determined the physical, biological, and immunological properties of the recombinant Stx2 B subunit. The results in the following report suggest this antigen might represent a potential component in an acellular STEC vaccine.

2. 2. Materials and Methods

2. 2. 1. Plasmid construction.

Standard techniques were used to construct the plasmids (Sambrook et al., 1989). All restriction enzymes were obtained from Gibco BRL, Life Technologies, Burlington, Ontario, Canada. Plasmids were isolated from the bacteria by the mini DNA preparation procedure described by Elango and colleagues (Elango et al., 1997). All constructs were characterized by PCR, restriction digested and sequenced, using the Sequenase Version 2.0 kit (United States Biochemical Corporation, Cleveland, Ohio).

The *stx1b* leader sequence was amplified by PCR from pVERO (obtained from Dr. Bruce Ritchie, University of Alberta, Canada), using primers 1 (5' gaa gga gat atg cat atg aaa a 3') and 2 (5' atc agc tgc agc cag cgc act tgc tga3'). The resulting 60bp fragment and pRSETB vector (Invitrogen, San Diego, California) were digested with Nde1 and Pst1 (sites introduced by primers) and ligated. Electrocompetent DH5α *E. coli* cells (Bethesda Research Laboratories, Gaithersburg, Maryland) were transformed and the resulting isolated plasmid was designated pSTX1BLS.

The *stx2b* gene, minus the leader peptide coding sequences, was amplified by PCR from chromosomal DNA isolated from *E. coli* C600 containing the 933W bacteriophage (Newland et al., 1985) (a gift from Dr. David Acheson, Department of Geographic Medicine, New England Medical Center, Boston, Massachusetts). Upstream, primer 3 (5' aat ggc tgc aga ttg cgc taa agg taa 3') and downstream, primer 4 (5' cag gtg gta cct cag tca tta tta aac 3') introduced restriction enzyme sites Pst1 and Kpn1, respectively. The 220bp fragment and pSTX1BLS were digested with Pst1 and Kpn1 and ligated. Electrocompetent DH5α *E. coli* were transformed and the isolated plasmid was designated pSTX2BSTX1BLS.

The *stx2b* gene, including its native leader peptide coding sequences, was amplified by PCR from chromosomal DNA isolated from *E. coli* C600 containing the bacteriophage 933W, using primers 5 (5' gag ata tac ata tga aga aga tgt tta tgg c 3') and 4 (same as above). The 280bp fragment and pRSETB were digested with restriction enzymes Nde1 and Kpn1. Electrocompetent DH5α *E. coli* were transformed and the isolated plasmid named pSTX2B.

2. 2. 2. Stx2 B expression.

pSTX2BSTX1BLS, pSTX2B and pRSETB were transformed into *E. coli* strain TOPP3™ (rif^{r} [F', proAB, lacI^{q}ZÄM15,Tn10(tet^{r})] km^{r}) (Stratagene, La Jolla, California). Isolated colonies were then grown with shaking at 225rpm at 30°C to mid exponential phase (A$_{600}$ of 0.6) in 5 mL Luria-Bertani broth (LB, Difco Laboratories, Detroit, Michigan) supplemented with 50 μg/mL ampicillin (Sigma-Aldrich, Oakville, Ontario, Canada). The cultures were subsequently heat-induced by doubling their volume with fresh LB pre-heated to 55°C. These cultures were then incubated at 42°C for an additional 30 minutes and grown another 4 hours at 37°C, with shaking at 225rpm. Seventy five μL aliquots of these bacterial cultures were collected by centrifugation. The resulting bacterial cell pellets were lysed in 25 μL of Laemmli sample buffer and analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-PROTEAN II cell system (Bio-Rad, Mississauga, Ontario, Canada). Protein bands from the resulting SDS gel were electrophoretically transferred, for 15 minutes at 0.5 A, using a Trans-Blot Cell (Bio-Rad), to a PVDF membrane (Millipore, Bedford, Massachusetts). The western blot was subsequently cut in half and the top half of the blot was probed with a 1/10,000 dilution of polyclonal rabbit anti-maltose binding protein (New England Biolabs Inc., Mississauga, Ontario, Canada). The bottom half of the blots was probed with a 1/1,000 dilution of polyclonal rabbit Stx2 B subunit-specific antiserum, raised from native B subunits purified from the holotoxin. This was followed with a 1/10,000 dilution of the secondary antibody, peroxidase-conjugated goat anti-rabbit IgG (Sigma Aldrich), and developed with the ECL™ western blotting detection kit (Amersham Pharmacia Biotech, Buckinghamshire, England), as per the manufacturer's instructions. The blot was exposed for 2 minutes to Super RX Fuji medical X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan).

2. 2. 3. Large scale isolation and purification of recombinant Stx2 B subunit.

The *E. coli* TOPP3™ pSTX2BSTX1BLS clone was grown to mid exponential phase (A_{600} of 0.6) at 30°C, with shaking at 225rpm, in 500 mL of ampicillin-supplemented (50 µg/mL) LB. These cultures were then heat-induced for Stx2 B subunit expression as described above. The Stx2 B subunit was purified using 15 g of Synsorb-Pk (Lemieux et al., 1975) as described by Mulvey *et al.* (Mulvey et al., 1998) with these minor modifications. The Stx2 B subunit was eluted from the Synsorb-Pk in 2 x 100 mL of 1 M urea, 0.25 M NaCl, 0.05 M Tris, pH 10.0, with shaking at 150 rpm at 37°C for 30 min. The eluted fractions were then filter-sterilized through 0.22 µm pore size Steritop™ filter units (Millipore) and concentrated 75 times using a stirred-cell filtration apparatus (Amicon, Beverly, Massachusetts) fitted with a YM-3 membrane (Millipore). Urea was removed from these samples by diluting them to their original volume with 0.05 M Tris, 0.25 M NaCl buffer (pH 10), minus the urea, and the concentration step was repeated. This filtration dialysis procedure was performed a total of 3 times. Protein yield was determined with the BCA Protein Assay Kit (Pierce, Rockford, Illinois) as per the manufacture's instructions.

2. 2. 4. Stx2 holotoxin preparation.

Stx2 holotoxin was purified from *E. coli* C600 933W (Newland et al., 1985). Affinity purification of Stx2 was accomplished using Synsorb-Pk (Lemieux et al., 1975) following the published procedure (Mulvey et al., 1998) and with the minor modifications detailed in the previous section.

2. 2. 5. Vero cytotoxicity assays of purified recombinant Stx2 B subunit and Stx2 holotoxin.

Purified Stx2 holotoxin and the Stx2 B subunit were assayed for toxicity in Vero cells following a procedure described earlier (Armstrong et al., 1991; Mulvey et al., 1998).

2. 2. 6. HPLC gel filtration of recombinant Stx2 B subunit.

Approximately 150 μg of purified Stx2 B subunit was injected at a flow rate of 0.4 mL/min into an SE-100117 BioPrep HPLC column (Bio-Rad), equilibrated with 0.15 M NaCl, 0.05 M Na/K buffer, pH 6.8. The column was calibrated using

Bio-Rad standard proteins (Bio-Rad). The elution profile was recorded at a wavelength of 280 nm.

2. 2. 7. ELISA binding assay with recombinant Stx2 B subunit.

The ELISA assays were done in triplicate. One hundred μL/well of a bovine serum albumin (BSA) glycoconjugate containing an average of 24 moles of 8 methoxycarbonyloctyl (MCO) Pk trisaccharide (αGal(1-4)βGal(1-4)βGlc) sequences per mole, was coated overnight at 4°C, in 96 well EIA/RIA microtiter plates (Costar, Cambridge, Massachusetts). The MCO Pk trisaccharide-BSA was a gift from Dr. David Bundle (Department of Chemistry, University of Alberta, Canada). Control wells were coated with unconjugated BSA. The plates were washed 5 times with PBS buffer, pH 7.3, containing 0.05% Tween (PBST) and blocked for 1 hour at room temperature with 200 μL/well of 2% BSA (Sigma Aldrich, Oakville, Ontario) in PBST. The plates were washed 5 times with PBST buffer, and 100 μL/well of 1/5 serial dilutions of Stx2 B subunit (or Stx2 holotoxin), starting at 10 μg/mL was added overnight at room temperature. The plates were then washed 5 times with PBST and 100 μL/well of a 1/100 dilution of polyclonal rabbit Stx2 B subunit antiserum was added for 1 hour at room temperature. The plates were washed 5 times with PBST and a 1/5000 dilution of peroxidase-conjugated goat anti-rabbit IgG was added for 1 hour at room temperature. The plate was finally washed 5 times with PBST and developed for 30 minutes at room temperature with 200 μL/well of 10 mM Citrate buffer, pH

4.2, 0.06% H_2O_2, 0.055% ABTS (Boehringer Mannheim, Indianapolis, Indiana). The resulting absorbance data were recorded using a Spectramax 340 microtiter plate reader set at a wavelength of 405nm.

2. 2. 8. Apoptosis assays with Stx2 B subunit and Stx2 holotoxin.

The Gb_3-expressing Burkitt's lymphoma cell line, Daudi, was purchased from the ATCC (catalog # CCL-213). The cells were cultivated in RPMI 1640 medium (Gibco BRL) supplemented with 1 mM glutamine and 10% heat-inactivated fetal bovine serum (FBS). The Burkitt's lymphoma cells (4.6 X 10^6 cells/mL) were cultivated for 18 h in the presence of either 5 μM Camptothecin (Sigma Aldrich), 10 ng/mL Stx2 , or 1 μg/mL Stx2 B. Apoptotic cells were labeled following the procedure described in the in situ cell death detection kit (Boehringer Mannheim) and then quantified by flow cytometry (fluorescence at 525 nm) using a FACscan flow cytometer (Becton Dickenson, San Jose, California).

2. 2. 9. Endotoxin quantification and removal from cloned Stx2 B subunit.

The endotoxin concentration in the Stx2 B subunit preparations was determined using the colorimetric Limulus amebocyte lysate (LAL) assay, QCL-1000, kit (BioWhittaker, Inc., Walkersville, Maryland) and recorded as endotoxin units/mL of protein solution. Excess endotoxin was removed from the purified Stx2 B subunit preparations using Detoxi-Gel™ (Pierce), according to the directions supplied with the product. The endotoxin content of 10 mg of Stx2 B subunit was reduced to 0.4 endotoxin units/mL (low endotoxin) by passing 0.2 mL of the sample 4 times through a 1 mL (bed volume) Detoxi-Gel column. The protein recovery was approximately 80% to 90% at each passage through the column.

2. 2. 10. Rabbit immunization procedures.

The rabbit experiments were conducted according to the guidelines of the Canadian Council for Animal Care using protocols approved by the University of Alberta Health Sciences Animal Welfare and Policy Committee. Eight female New Zealand white rabbits (Vandermeer Rabbitry, Edmonton, Alberta, Canada) were obtained at a weight of approximately 2 kg each and divided into two groups of four. The rabbits in one group were injected in the sub-scapular region with the cloned low endotoxin Stx2 B subunit preparation homogenized in an equal volume of Quil-A™ Saponin (Cedarlane® Laboratories Ltd., Hornby, Ontario, Canada) adjuvant. The rabbits in the second group were sham-immunized with a 1:1 homogenate of Quil-A and pyrogen-free 0.9% NaCl irrigation solution, USP (Baxter Corporation, Toronto, Ontario, Canada). The rabbits were injected three times, on a monthly schedule, the first time receiving 150 μg of antigen and each of the subsequent times, 100 μg of antigen each.

The eight rabbits were then sub-grouped for a second round of immunization. Four rabbits, two from the low endotoxin Stx2 B subunit-immunized group and two from the sham-immunized control group, received two additional injections of 100 μg of a cloned Stx2 B subunit preparation in which the endotoxin concentration had only been reduced to 2000 endotoxin units/mL (high endotoxin Stx2 B subunit preparation). The remaining four rabbits, two of those previously immunized with the low endotoxin Stx2 B subunit preparation and two from the sham-immunized control group received two additional injections of 100 μg of the low endotoxin Stx2 B subunit preparation. Table 1 summarizes the immunization schedule for each of the rabbits.

2. 2. 11. Analysis of rabbit sera by the western immunoblot procedure.

Sera were drawn from the ear vein of each rabbit. Pre- and post-immunization sera were then analyzed for specific antibody production by the western immunoblot procedure. SDS-PAGE of Stx2 holotoxin was performed, and proteins from the resulting SDS gels were electrophoretically transferred to PVDF membranes. The immunoblots were then probed with a 1/5,000 dilution of each of the rabbit sera followed by a 1/10,000 dilution of peroxidase-conjugated goat anti-rabbit IgG. The immunoreactive protein bands were detected using the ECL™ western immunoblot detection kit with a 1 minute exposure to Super RX Fuji medical X-ray film.

2. 2. 12. Analysis of rabbit sera in the Verocytotoxicity neutralization assay.

Rabbit sera were serially diluted in pH 7.3 PBS in 96 well microtiter plates in duplicate. Five μL of 2 μg/mL Stx2 holotoxin in PBS was mixed with 75 μL of each of these serial dilutions. After 1 h incubation at 37°C, 20 µL from each well in the dilution plates was transferred to the corresponding well of a 96 well plate containing confluent Vero cell monolayers and 200 µL of MEM supplemented with 5% FBS (Gibco BRL, Life Technologies, Grand Island, New York). After 2 hours, the MEM was emptied from the Vero cell microtiter plates. Each of the monolayers was washed twice with 200 µL/well of MEM, fresh MEM plus 5% FBS was added to the wells and the plates were incubated for an additional 46 hours at 37°C in an atmosphere of 5% CO_2/95% air. The Vero cells were then fixed with methanol and Giemsa stained (Fisher, Pittsburgh, Philadelphia). The results were recorded using a Spectramax 340 microtiter plate reader set at a wavelength of 620nm and the data analyzed as described previously (Armstrong et al., 1991).

2. 2. 13. Determination of antibody titers by ELISA.

IgG titers in the rabbit sera were determined in triplicate by ELISA. Two hundred µL/well of 2.5 µg/mL Stx2 was coated overnight at 4°C, in 96 well EIA/RIA plates. The plates were then washed 5 times with PBST and blocked with 2% BSA, as described earlier. Two hundred µL of serial dilutions of sera in 2% BSA, PBST was added to the wells and the plates were incubated for 2 hours. The plates were next washed 5 times with PBST and 200 µL/well of a 1/5000 dilution of peroxidase-conjugated goat anti-rabbit IgG, was added for 1 hour at room temperature. The plates were washed 5 times with PBST, developed with ABTS, and the results were recorded at 405nm as described earlier.

2. 2. 14. Rabbit Stx2 holotoxin challenge experiments.

Rabbits were challenged with 5 μg of Stx2 holotoxin/kg of body weight (Fujii et al., 1996). The purified Stx2 holotoxin preparations were homogenized with an equal volume of Quil-A adjuvant and injected into the sub-scapular region of each rabbit. The rabbits were then monitored every four hours for 1 week, and once daily for a period of 3 weeks thereafter. The rabbits were euthanized as soon as toxic effects (anterior ataxia or paralysis), were observed. At the end of the one month study, asymptomatic surviving rabbits were also euthanized for postmortem examination.

Post-mortem, and micropathology studies were done on the brain, liver, spleen, kidneys, jejunum, cecum, and colon of each rabbit. These organs were removed and placed in 10% neutral buffered formalin for fixation. After 24-48 hours in the fixative solution, samples were trimmed into tissue cassettes, processed into paraffin blocks, thin sectioned, stained with hemataxylin and eosin and mounted on glass microscope slides using standard techniques. A board certified veterinary pathologist then examined the sections.

2. 3. Results

2. 3. 1. Expression of recombinant Stx2 B subunit.

Western immunoblotting analysis of whole cell lysates of the TOPP3™ *E. coli* clones containing the plasmid constructs, pSTX2BSTX1BLS or pSTX2B, revealed that only the clone containing pSTX2BSTX1BLS (*stx2b* with the *stx1b* leader sequence) expressed an immunoreactive protein that co-migrated with the Stx2 B subunit band in the positive control lane (Figure 2-1, lane 2, bottom blot) containing Stx2 holotoxin. *E. coli* isolates containing the empty expression vector, pRSETB, or the vector containing the native Stx2 B subunit insert (pSTX2B, *stx2b* with its native leader sequence) failed to express detectable levels of the Stx2 B subunit. The immunoblots presented in Figure 2-1 also demonstrated that the *E. coli* TOPP3™ pSTX2BSTX1BLS strain only expressed detectable levels of Stx2 B subunit after heat-induction. The top half of the western immunoblot shown in Figure 2-1 was probed with antibodies to constitutively expressed maltose binding protein. These results demonstrated that equal amounts of extracts from each of the *E. coli* strains analyzed by this procedure were applied to the SDS-PAGE lanes.

2. 3. 2. Purification of the Stx2 B subunit.

Expression and purification of the Stx2 B subunit was performed using the TOPP3™ pSTX2BSTX1BLS *E. coli*. The average yield of purified Stx2 B

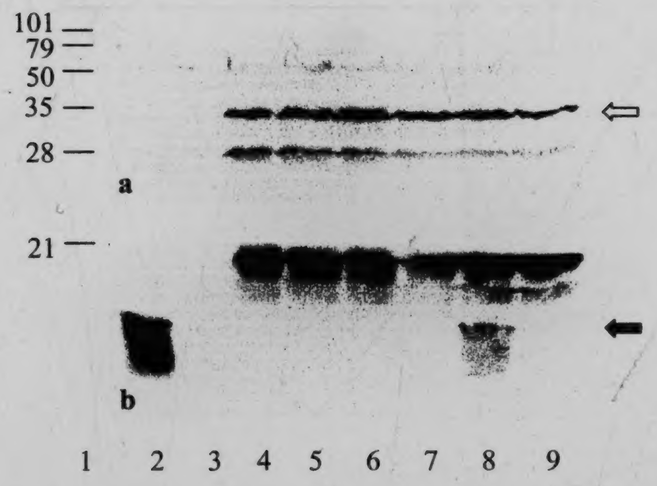

Figure 2-1. Western immunoblot analysis of whole cell lysates comparing the expression of recombinant Stx2 B subunit in the different TOPP3™ *E. coli* constructs. The distance migrated by each of the pre-stained calibration standards in lane 1 is indicated by their respective molecular weights (x 10^{-3}) on the left side of the figure. Lane 2 contained purified Stx2 holotoxin. Lane 3 is a blank lane. Lanes 4, 5 and 6, contained the uninduced whole cell lysates of TOPP3™ pRSETB, TOPP3™ pSTX2BSTX1BLS, and TOPP3™ pSTX2B respectively. Lanes 7, 8 and 9 contained samples of post heat-induction whole cell lysates of TOPP3™ pRSETB, TOPP3™ pSTX2BSTX1BLS, and TOPP3™ pSTX2B respectively. The top half (a) of the western immunoblot was reacted with a primary antibody specific for the constitutively expressed *E. coli* maltose binding protein (open arrow) and the bottom half (b) of the western blot was probed with rabbit Stx2 B subunit-specific polyclonal antiserum (closed arrow).

protein from 12 independent 4 L preparations was 0.7 mg/L (range 1 to 0.3 mg/L). This yield compares favorably with the 0.1mg/L yield obtained in the procedure described by D. Acheson and colleagues (Acheson et al., 1995). SDS-PAGE analysis of the purified Stx2 B subunit is shown in Figure 2-2. Densitometry analysis of the protein bands on the SDS polyacrylamide gels revealed a purity of >95% in the Synsorb-Pk-purified Stx2 B preparations.

2. 3. 3. Vero cytotoxicity assay on Stx2 B subunit and Stx2 holotoxin.

It was reported previously that recombinant Stx1 B subunit is non-toxic (Acheson et al., 1993; Ramotar et al., 1990) and does not induce apoptosis in Vero cells (Williams et al., 1997). We therefore studied the Vero cytotoxicity of our recombinant Stx2 B subunit preparations and purified Stx2 holotoxin. The CD_{50} of purified Stx2 holotoxin in Vero cells was 38 pg/mL (Figure 2-3). This was consistent with previously published Stx2 CD_{50} values in Vero cells (Acheson, 1993; Mulvey et al., 1998). The recombinant Stx2 B subunit was non-toxic in the Vero cytotoxicity assay at up to 5 μg/mL (Figure 2-3). These results are consistent with previously published results for the cloned Stx1 B subunit (Acheson et al., 1993; Ramotar et al., 1990).

2. 3. 4. Molecular mass estimation of the Stx2 B subunit.

The molecular mass of the recombinant Stx2 B subunit was determined by HPLC gel filtration chromatography (Figure 2-4). The Stx2 B subunit eluted

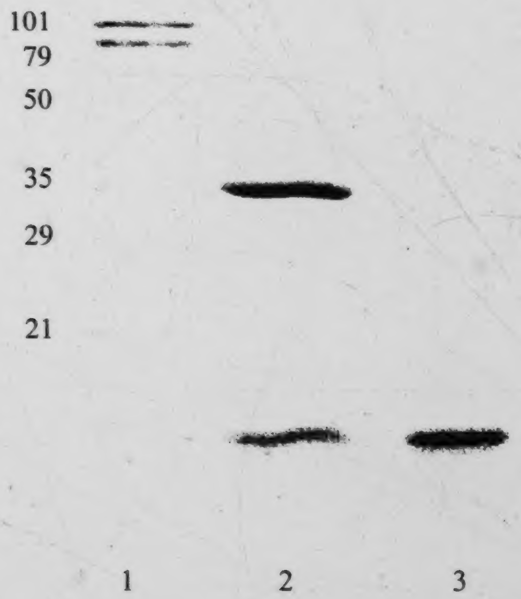

Figure 2-2. 15% SDS-PAGE reducing gel of purified recombinant Stx2 B subunit. Lane 1, prestained low molecular weight standard proteins (molecular weights x 10^{-3} to the left of the lane). Lane 2, purified Stx2 holotoxin. Lane 3, purified recombinant Stx2 B subunit. The gel was stained with Coomassie blue.

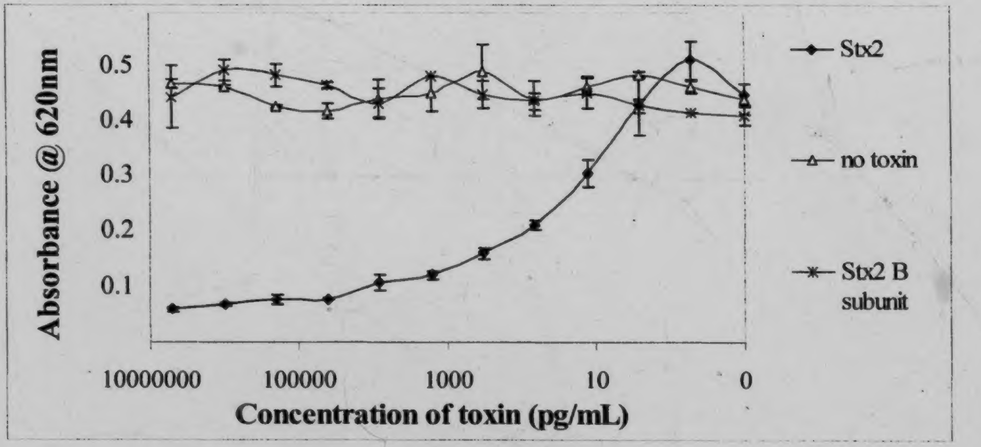

Figure 2-3. Assay comparing the Verocytotoxicities of purified Stx2 holotoxin and the recombinant Stx2 B subunit (n =2 and the error bars represent standard error).

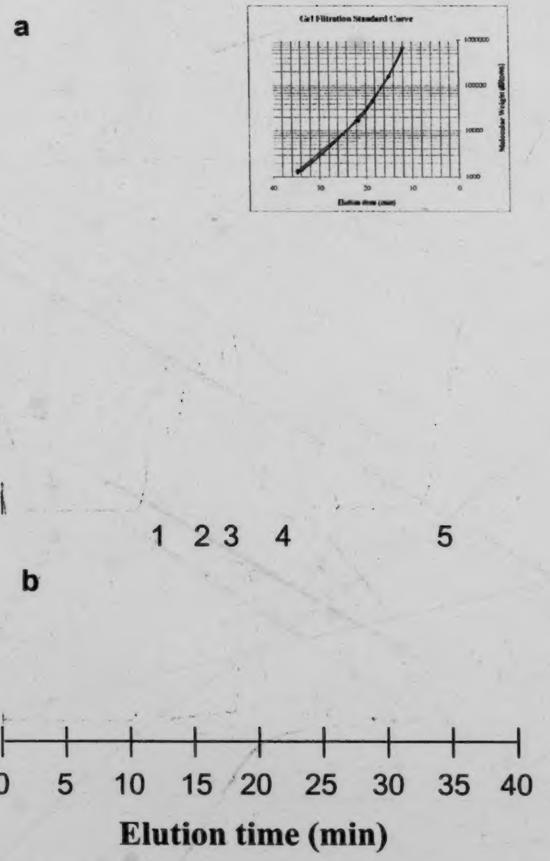

Figure 2-4. Molecular weight estimation of recombinant Stx2 B subunit by HPLC gel filtration chromatography. a. Bio-rad gel filtration standard (900ug), peak 1, Thyroglobulin 670kD; peak 2, Gamma globulin 158kD; peak3, Ovalbumin 44kD; peak 4, Myoglobin 17kD; peak 5, Vitamin B-12 1.35kD. b. Stx2 B subunit, elution time 19.5 min, estimated MW 38kD.

from the calibrated HPLC column as one sharp symmetrical peak with a calculated molecular weight of 38 kDa. This value closely approximates the theoretical MW (39,085 Da) of the Stx2 B pentamer.

2. 3. 5. Stx2 B subunit binding to MCO Pk-BSA.

The ELISA data presented in Figure 2-5 revealed that, the cloned Stx2 B subunit bound to a receptor analog in a similar manner to Stx2 holotoxin.

2. 3. 6. Apoptosis assay using Burkitt's lymphoma cells.

The TUNEL procedure was used to determine if the cloned Stx2 B subunit induced apoptosis in Gb_3-expressing Daudi Burkitt's lymphoma cells (Figure 2-6). Stx2 holotoxin was also tested for comparison. Camptothecin, a known apoptogenic agent, induced apoptosis at a concentration of 5 μM. Stx2 induced apoptosis in 20% of the Daudi cells at a concentration of 10 ng/mL. This was similar to published apoptosis results for Stx1 holotoxin (Mangeney et al., 1993). However, unlike the Stx1 B subunit (Mangeney et al., 1993), the Stx2 B subunit did not induce apoptosis at up to 100 fold this concentration (1 μg/mL).

2. 3. 7. Low endotoxin Stx2 B subunit as an immunogen in rabbits.

The endotoxin concentration (>1,000,000 units/mL) in the Synsorb-Pk-purified Stx2 B subunit preparations was considered unacceptable for vaccine preparations. Therefore, polymyxin-B-agarose (Detoxi-Gel) affinity

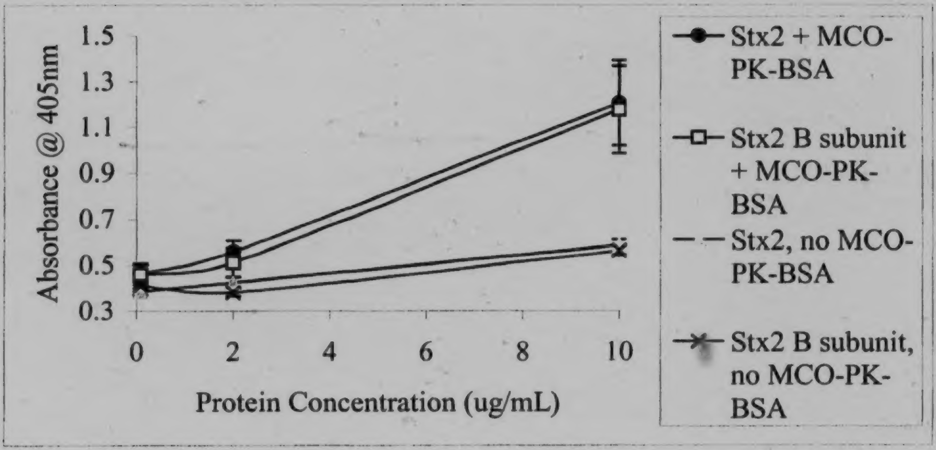

Figure 2-5. ELISA confirming the recombinant Stx2 B subunit receptor binding activity. Plates were coated with or without the MCO-Pk-BSA analogue. Bound Stx2 holotoxin or Stx2 B subunit was detected with a rabbit Stx2 B subunit polyclonal antiserum, followed by peroxidase conjugated anti-rabbit IgG. Error bars represent the standard deviation (n = 3).

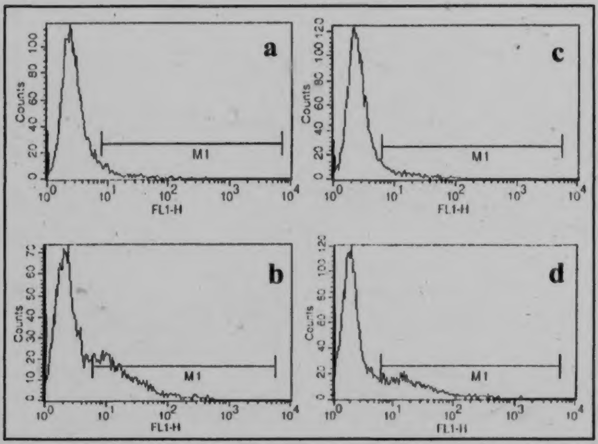

Figure 2-6. Flow cytometry analysis of TUNEL labeled Burkitt's lymphoma cells post treatment with Stx2 or recombinant Stx2 B subunits. Treatments were performed in duplicate samples, with only one of the samples illustrated. a. no treatment (4.03% apoptotic). b. 5uM camptothecin (29.05% apoptotic). c. 1ug/mL Stx2 B subunit (5.44% apoptotic). d. 10ng/mL Stx2 holotoxin (20.47% apoptotic).

chromatography was used to reduce the endotoxin concentration to <0.4 units/mL in these antigen preparations. Although four rabbits were immunized three times over a period of three months with the low endotoxin Stx2 B preparation, western immunoblot analysis revealed no evidence of Stx2 B subunit-reactive antibodies in sera obtained from these animals (results not shown). As well, none of the sera from these low endotoxin Stx2 B subunit-immunized animals, even at the lowest dilution tested (1/20), protected Vero cells from a cytolethal dose of Stx2 holotoxin (data not shown). However, ELISA data indicated that, despite the negative western immunoblot and Verocytotoxicity neutralization assay results, some anti-Stx2 antibodies were being produced in the immunized animals. As anticipated, none of the pre-immunization sera from all eight rabbits nor any sera from the four sham-immunized control animals contained evidence of Stx2-reactive antibodies by ELISA, immunoblot, or Verocytotoxicity neutralizing assays (results not shown).

2. 3. 8. Immunization with a high endotoxin Stx2 B subunit preparation.

Endotoxin is a known immunogen and has adjuvant properties (Johnson, 1985; Morrison and Ryan, 1979). We therefore thought the presence of some endotoxin, may augment the rabbit immune response to the Stx2 B subunit. To test this theory, two of the rabbits previously immunized (primed) with the low endotoxin Stx2 B preparation were injected an additional 2 times with a partially detoxified (high endotoxin) Stx2 B subunit preparation containing approximately

2000 endotoxin units/mL (Table 2-1). The other two rabbits from this group were injected 2 more times with the same low endotoxin Stx2 B preparation they received in the first round of immunization. The four control rabbits from the first round of immunization were injected two more times with either the low (2 rabbits) or high endotoxin (2 rabbits) Stx2 B subunit preparations (Table 2-1).

Western immunoblot analysis of sera following this second round of immunizations revealed that rabbits K106 and K110, which had been primed with three injections of the low endotoxin Stx2 B subunit preparation and then received an additional two injections of high endotoxin (approximately 2000 units/mL) Stx2 B subunit, developed a specific antibody response to the immunogen after the first of the two additional injections (Figure 2-7, panel A). As well, after receiving two injections of the low endotoxin Stx2 B preparation, one of the first round, sham-immunized control rabbits, K103, produced a specific antibody response to the Stx2 B subunit (Figure 2-7, panel B). Rabbit K107, another of the first round sham-immunized animals, produced a weak antibody response (Figure 2-7) to the Stx2 B subunit after being injected twice with the high endotoxin Stx2 B subunit preparation.

Verocytotoxicity neutralization assays demonstrated that the second round hyper-immune sera from rabbits K106 and K110 protected Vero cells from a lethal dose of Stx2 holotoxin at up to 1/160 and 1/40 dilutions, respectively (results not shown). However, despite western immunoblot evidence of Stx2 B subunit-specific reactivity in the post-immunization sera from rabbits K103 and

	Rabbits immunized:		
Rabbit ID No.	3 Times low endotoxin Stx2 B	2 Times low endotoxin Stx2 B	2 Times high endotoxin Stx2 B
K101 (antibody titer)	-	+ (1/625)	-
K103[*]	-	+ (1/625)	-
K104	+ (1/4000)	+ (1/3125)	-
K105	+ (1/1000)	+ (1/625)	-
K106[†]	+ (1/12500)	-	+ (1/15625)
K107	-	-	+ (1/4000)
K110[†]	+ (1/2500)	-	+ (1/2000)
K111	-	-	+ (1/2000)

Table 2-1. Rabbit immunization schedule and resulting ELISA antibody titers to Stx2 holotoxin.

[*] Western immunoblot-positive for Stx2 B-specific antibody but Stx2 holotoxin Verocytotoxicity neutralization-negative.

[†] Western immunoblot-positive for Stx2 B-specific antibody and Verocytotoxicity neutralization-positive for Stx2 holotoxin.

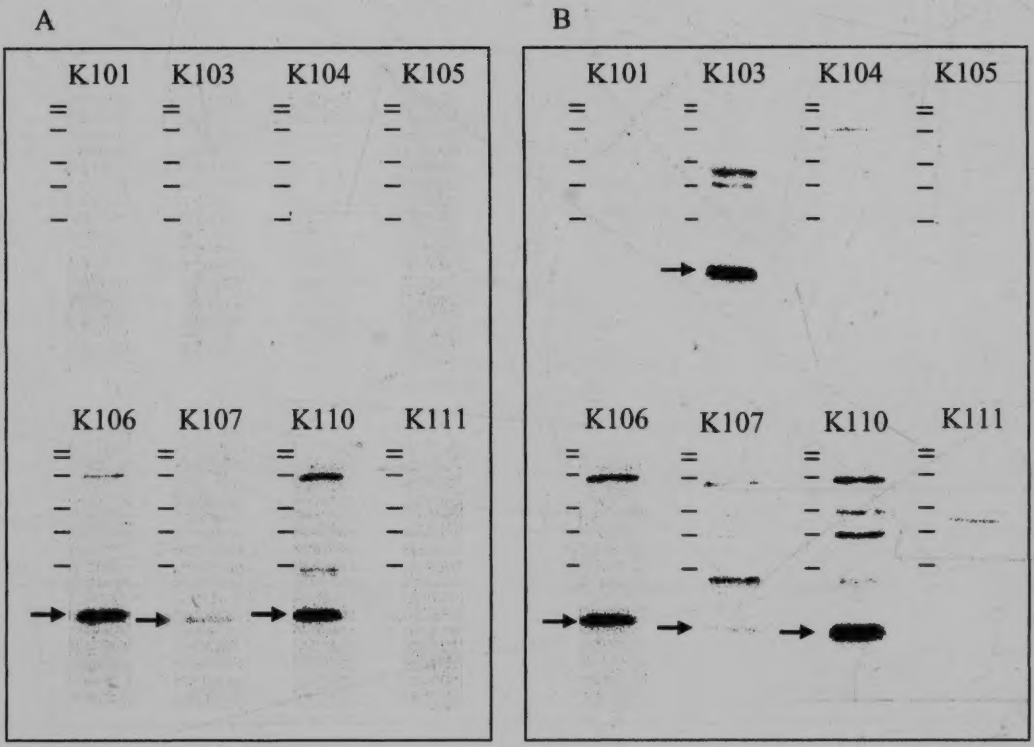

Figure 2-7. Western immunoblots demonstrating Stx2 B subunit antibody production in rabbits. Purified Stx2 holotoxin, containing both A and B subunits, was transferred to each immunoblot and probed with sera from each of the rabbits, followed by peroxidase conjugated anti-rabbit IgG. Low molecular weight pre-stained standard proteins from Bio-Rad were used to calibrate the gels. The lines adjacent to each of the blots indicate the distance migrated by the pre-stained protein standards. Panel A. Immunoblots using sera obtained after the first of the second round injection of antigens. Panel B. Immunoblots using sera obtained after the second round injection of antigens. The arrow indicates the position of the Stx2 B subunit.

K107, these sera were not neutralizing in the Verocytotoxicity assay. Also, the ELISA antibody titers of the sera from all the immunized rabbits (Table 2-1) did not correlate with either the endotoxin content of the Stx2 B subunit preparations or the western immunoblot or Verocytotoxicity protection results.

2. 3. 9. Rabbit challenge with Stx2 holotoxin.

All the Stx2 holotoxin-challenged rabbits that failed to display western immunoblot evidence of Stx2 B subunit-specific antibodies developed Stx2-related symptoms between post-challenge days 2 and 4 and were euthanized (Figure 2-8). Rabbit K107, which developed a weak western immunoblot response to the Stx2 B subunit, also developed Stx2-related symptoms on post challenge day 2 and was euthanized. In contrast, rabbits K103, K106 and K110, which produced western immunoblot positive Stx2 B subunit antibodies, remained asymptomatic throughout the 1 month study. Post-mortem examination showed that all the unprotected rabbits displayed varying degrees of Stx-mediated organ and tissue damage. In contrast, all tissues and organs in each of the protected rabbits, K103, K106, and K110 appeared normal (data not shown).

We noted that the Stx2 B subunit band was not the only immunoreactive band visible in the western immunoblots presented in Figure 2-8. The pattern of these non-Stx2 B subunit immunoreactive bands also varied between serum samples from the different rabbits, and were less intense than the Stx2 B subunit immunoreactive band. These non-Stx2 B subunit immunoreactive bands could

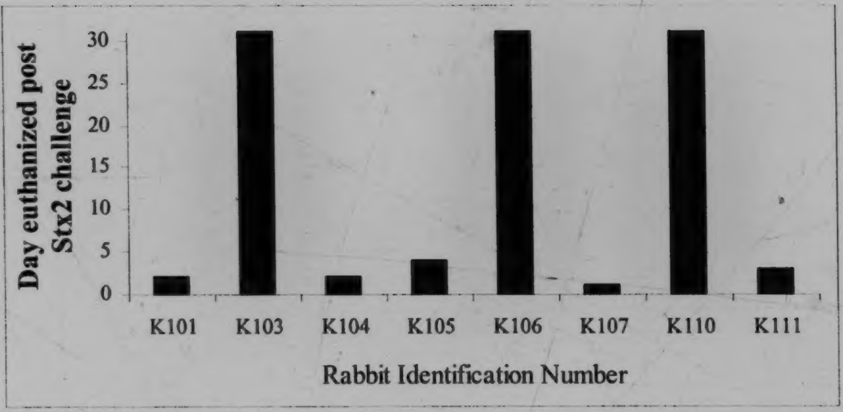

Figure 2-8. Fate of rabbits challenged with a lethal dose of Stx2 holotoxin. Rabbits were euthanized when obvious signs of Stx2 holotoxin-mediated symptoms (anterior ataxia and/or diarrhea) became apparent. Rabbits K103, K106 and K110 remained healthy and were euthanized for micropathological examination at the end of the month study.

have resulted from antibodies specific to minor impurities in the immunogen preparations. Alternatively, the Stx2 B subunit-specific antibodies may have recognized SDS-resistant polymerized forms of the Stx2 B subunit on the western immunoblots. Regardless, the only common immunoreactive band in the western immunoblots prepared with sera from all of the protected rabbits (K103, K106 and K110) was the Stx2 B subunit band.

2. 4. Discussion

By substituting the native peptide leader sequence of the Stx2 B subunit with that of the Stx1 B subunit we were able to obtain highly purified, apparently pentameric, native Stx2 B subunits in the TOPP3™ *E. coli* expression strain. The cloned Stx2 B subunit was purified to near homogeneity (>95% purity) by affinity batch purification using Synsorb-Pk, a compound consisting of an insoluble matrix containing covalently linked Stx2 trisaccharide receptor analogs (Lemieux et al., 1975; Mulvey et al., 1998) (Figure 2-5). This implied that the Stx2 B subunits retained their ability to bind to receptors. This activity was confirmed by the ELISA results presented in Figure 2-5.

Since the recombinant Stx2 B subunit might be used in a potential acellular EHEC vaccine, any evidence of biological activity in this recombinant Stx2 B subunit would be of concern. Previously, it was reported that a recombinant Stx1 B subunit preparation induced apoptosis in Burkitt's lymphoma (Mangeney et al., 1993) and astrocytoma cells (Arab et al., 1998). However, there are conflicting views on the apoptotic activity of the Stx1 B subunit. I. M. Nakagawa and colleagues recently showed that HeLa cells transfected with the *stx1b* gene undergo apoptosis (Nakagawa et al., 1999). In contrast, S. B. Calderwood and colleagues reported that recombinant Stx1 B subunit is non-toxic to HeLa cells at a concentration up to 10 μg/mL (Calderwood et al., 1990). J. M. Williams and colleagues showed that the Stx1 B subunit does not induce apoptosis in Vero cells (Williams et al., 1997). More recent studies showed that the Stx A subunit, not the B subunits, is essential for activating cellular proteins thought be involved in the apoptosis signaling cascade (Kojima et al., 2000; Suzuki et al., 2000). We therefore tested the apoptotic activity of our cloned Stx2 B subunit and found it did not induce apoptosis in Burkitt's lymphoma cells.

Stx2 holotoxin, in comparison to Stx1 holotoxin, has a lower affinity for its receptor (Head et al., 1991). Structural studies of the Stx1 B pentamer also reveal that each monomer protein contains 3 possible functional carbohydrate receptor binding sites (Ling et al., 1998). In contrast, studies on a mutant Stx2e B subunit show that the third site in this variant Stx may not be functional in binding Gb_3 (Ling et al., 2000). Experiments with chimeric Stx1/Stx2 proteins revealed that the different cytotoxic effects of the two toxins is specific to their B subunits (Head et al., 1991). Therefore, there are a number of possible explanations for why our cloned Stx2 B subunit, unlike the Stx1 B subunit, failed to induce apoptosis in Burkitt's lymphoma cells.

In the first round of immunization, the lack of an apparent immune response to the Stx2 B subunit might have been due to insufficient augmentation of the rabbit immune system by the Quil-A adjuvant. Endotoxin, or lipopolysaccharide (LPS) has known adjuvant properties, stimulating both humoral and cell mediated immunity (Johnson, 1985; Morrison and Ryan, 1979). Therefore, the hypo-reactivity of the rabbit immune system to the low endotoxin Stx2 B subunit may have been due to the lack of adjuvant activity in this antigen preparation. Our finding that endotoxin may have stimulated an immune response to the cloned Stx2 B subunit is consistent with this hypothesis. It is also possible however, that endotoxin in the antigen preparations may have sufficiently primed the rabbit immune system such that the challenge antigen, Stx2 holotoxin, was able to rapidly generate a protective immune response, perhaps directed at the immunodominant A subunit. We feel, however, the results in rabbit K103, which survived the Stx2 holotoxin challenge after receiving only the low endotoxin Stx2 B subunit preparation, run counter to this alternative explanation for the role of endotoxin in this system.

Western immunoblotting and Verocytotoxicity neutralization assays revealed that rabbits K106 and K110, developed Stx2 B subunit-specific antibodies after receiving additional injections of high endotoxin Stx2 B subunit. Western immunoblot analysis also revealed a Stx2 B subunit-specific antibody response in rabbit K103, which received only two injections of the low endotoxin Stx2 B subunit. However, the serum from this animal did not neutralize Stx2 holotoxin cytotoxicity in Vero cells. This result underscores the potential for population-level differences in the quality of an immune response to immunogens, such as the low endotoxin Stx2 B subunit preparation. Such differences may also be apparent in the lack of correlation between the ELISA results and variable patterns of western immunoblot-reactive bands in sera from animals receiving identical immunogen preparations (Figure 2-1 and Table 2-1).

The data in Figure 2-7, panel A, indicate that rabbits K106 and K110, which were primed by three injections of the low endotoxin Stx2 B subunit, produced an anti-Stx2 B subunit immune response after only one injection of the high endotoxin Stx2 B subunit preparation. Unlike these primed rabbits, the two animals that received only two injections of high endotoxin Stx2 B subunit (K107 and K111) did not develop (K111) or developed only a weak (K107) antibody response to the Stx2 B subunit. This suggests that simply including endotoxin in the Stx2 B subunit preparation was not sufficient to induce a protective immune response after only two injections of immunogen.

Previously, D. Acheson and colleagues evaluated several Stx2 B subunit cloning and expression strategies (Acheson et al., 1995). These included expressing the Stx2 B subunit behind the T7 or tac promoters in *E. coli* and *V. cholerae* as well as constructing an Stx2 B subunit-maltose binding protein fusion. Although, D. Acheson and colleagues were able to produce anti-Stx2 B subunit antibodies in rabbits, the antisera were not protective in tissue culture cell neutralization assays, nor did they proceed to test the immunoprophylactic activity of their cloned antigens *in vivo* (Acheson et al., 1995). They attributed the lack of cytotoxicity neutralization to pentamer stability problems. It is possible that had this group (Acheson et al., 1995) challenged their immunized rabbits with Stx2 holotoxin, they may have survived, akin to our rabbit K103 whose post-immune serum was Stx2 B subunit immunoblot positive and Verocytotoxicity neutralization negative.

While high concentrations of endotoxin are unacceptable in vaccines, there are alternatives that could be included with the Stx2 B subunit. With the advent of new-generation multi-component vaccines, including recombinant subunit, and synthetic peptide vaccines, eliciting a protective immune response to these purified antigens has proven to be challenging, and the search for safe adjuvants has become an essential part of this enterprise (Singh and O'Hagan, 1999). Human trials are currently underway to increase the number of approved adjuvants, and overcome the difficulties involved with the immunoreactivity of some of the new-generation vaccine formulations. These experimental adjuvants include, among others, the detoxified LPS molecule, monophosphoryl lipid A (MPL) (Schneerson et al., 1991; Ulrich and Myers, 1995), and a saponin derivative, QS21 (Newman et al., 1992; Wu et al., 1992). Ongoing work in our lab is presently directed at determining which of the new generation adjuvants may be used most effectively to enhance the protective properties of the cloned Stx2 B subunit in future potential STEC vaccines. Nonetheless, with this present study, we have demonstrated that it is possible to generate an active Stx2 B subunit-specific protective immune response in animals. Our results elaborate on the work by Nakao and colleagues, who previously reported on the immunotherapeutic potential of Stx2 B subunit-specific antibodies (Nakao et al., 1999).

Chapter 3

Development of a Shiga toxin 2 B subunit conjugate-based vaccine

A version of this chapter has been submitted to *Vaccine*. Marcato, P., Mulvey, G. L., Wee, S. S. Y., Griener, T. P., Armstrong, G. D.

G. L. Mulvey contributed intellectually and helped monitor animals during challenge experiments. S. S. Y. Wee and T. P. Griener helped conduct the ELISA and immunoblot assays, and also helped monitor animals during the challenge experiments.

This work was supported by operating grants MWS 56081 from the Canadian Institutes for Health Research (CIHR) and VP 17 from the Canadian Bacterial Diseases Network to GDA.

Figures 3-8 appeared as "data not shown" in the version of this chapter submitted to *Vaccine*.

In this chapter, I show how adjuvant formulation and conjugation to a carrier protein can be used to overcome the hypoimmunogenicity of the recombinant Stx2 B subunit described in Chapter 2.

3. 1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) cause hemorrhagic colitis (HC) and, in 10 to 15% of infected subjects, hemolytic-uremic syndrome (HUS) (Karmali, 1989). HUS is characterized by acute renal failure, hemolytic anemia, and thrombocytopenia. EHEC are also sometimes referred to as Shiga toxin-producing *E. coli* (STEC) since the production and release of Shiga toxins (Stx) is critical for their full virulence (Strockbine et al., 1986). Once EHEC has colonized the gut, it is possible for Stx to be released and spread from the intestine by a number of possible mechanisms (Acheson et al., 1996b; Hurley et al., 2001) into the circulatory system, allowing the toxins to reach target tissues and organs.

There are two immunologically distinct families of Stx, Stx1 and Stx2, associated with EHEC serotypes isolated from human subjects (Lingwood, 1996). These toxins display a classic AB_5 structure (Fraser et al., 1994; Stein et al., 1992). Five Stx B subunits form a pentamer that recognizes globotriaosylceramide (Gb_3) receptors found on many eukaryotic cell surfaces (Jacewicz et al., 1986). Upon receptor ligation, the toxin is internalized by the host cell and the A subunit's RNA *N*-glycosidase activity becomes activated, resulting in the removal of a specific adenine from the eukaryotic 28S rRNA component of the 60S ribosomal subunit (Endo et al., 1988; Reisbig et al., 1981). This Stx A subunit-mediated rRNA depurination activity causes eukaryotic cell death by triggering apoptosis.

Epidemiological evidence suggests a close association between Stx2 expression by EHEC and a more severe course of illness in humans (Hashimoto et al., 1999; Kleanthous et al., 1990; Ostroff et al., 1989; Scotland et al., 1987). Stx2 is also more lethal than Stx1 in mice (Tesh et al., 1993; Wadolkowski et al., 1990) and baboons (Siegler et al., 2003) and causes greater intestinal pathology in rabbits (Head et al., 1988). As well, the universal expression of Stx1 and/or Stx2 by virtually all EHEC serotypes makes the toxins attractive acellular vaccine components. Previously, we assessed the immunoprophylactic potential of a purified recombinant Stx2 B subunit preparation in an adult rabbit Shiga toxemia model (Marcato et al., 2001). We showed therein that the immunized animals were protected from a subsequent challenge with a lethal dose of Stx2 holotoxin. However, effective vaccination in the rabbit study was found to be unpredictable and required the undesirable presence of endotoxin (Lipopolysaccharide [LPS]) as an adjuvant.

Adjuvants have a great influence on the characteristics of the immune response to a particular antigen (Audibert and Lise, 1993). Ideally, an adjuvant should induce specific long-lasting protective immunity even with weakly immunogenic antigens, be effective in children and adults, chemically defined, non-reactogenic, and non-immunogenic (Gupta and Siber, 1995). Herein, we have extended our previous immunoprophylaxis study by evaluating the recombinant Stx2 B subunit as well as the Stx2 B subunit conjugated to Keyhole limpet hemocyanin (KLH), combined with several acceptable adjuvants, as potential vaccine preparations for protecting humans from the Stx-mediated complications of enterohemorrhagic *E. coli* infections.

3. 2. Materials and Methods

3. 2. 1. Toxin purification and cell lines.

Stx2 B subunit and Stx2 holotoxin were expressed and purified as described previously (Marcato et al., 2001; Mulvey et al., 1998). Endotoxin was removed from all the preparations using a Detoxi-gel (Pierce, Rockford IL) LPS affinity column as described by the manufacturer and in our previous article (Marcato et al., 2001). The colorimetric Limulus amebocyte lysate assay (QCL-100; BioWhittaker, Walkersville, MD) indicated that the endotoxin content of the purified Stx and Stx B pentamer preparations was < 1 endotoxin unit/mL. Primary normal human mesangial cells (NHMC), renal proximal tubule epithelial cells (RPTEC), human renal cortical epithelial cells (HRCE) and human microvascular endothelial cells (HMVEC-d) were purchased from BioWhittaker. The NHMC cells were cultivated in Mesangial Cell Growth Medium (MsGM), the RPTEC and HRCE cells were cultivated in Renal Epithelial Cell Growth Medium (REGM), and the HMVEC-d cells were cultivated in Microvascular Endothelial Cell Growth Medium-2 (EGM-2-MV) (BioWhittaker). Ramos Burkitt's lymphoma B cells were generously provided by Dr. Andrew Shaw (Cross Cancer Institute, University of Alberta). These cells were cultivated in

RPMI medium (Gibco BRL, Life Technologies, Burlington ON, Canada) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10 mM Hepes and 10% heat inactivated fetal bovine serum (FBS).

3. 2. 2. Conjugation of Stx2 B subunit to Keyhole limpet hemocyanin (KLH).

Imject® Mariculture KLH, high purity research grade (Pierce Biotechnology, Rockford, IL) was conjugated to the Stx2 B subunit using 1-Ethyl-3-(-3-Dimethylaminopropyl) carbodiimide Hydrochloride (EDC, Pierce Biotechnology) as per the manufacture's instructions. Briefly, 2mg of Stx2 B subunit was admixed with 2mg of KLH in conjugation buffer (0.1M 2-[N-morpholino] ethane sulfonic acid (MES), 0.1M NaCl, pH 4.7) with 250 μg of EDC and incubated at room temperature for 2 hours. To remove excess EDC, the conjugation reaction was exhaustively dialyzed at 4°C over a period of 4 days against 0.05M $NaPO_4$ buffer (pH 7.2) containing 0.15M NaCl using a 300 KDa MWCO membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA).

3. 2. 3. Mouse immunization.

These experiments were conducted in a randomized manner and adhering to the recommendations of the Canadian Council on Animal Care. The adjuvants tested in the study were Quil-A™ Saponin (Cedarlane® Laboratories Ltd., Hornby, Ontario, Canada), Ribi Adjuvant System (RAS) with synthetic Trehalose Dicorynomycolate (TDM) (Cedarlane), RAS with TDM and Monophosphoryl lipid A (MPL®, Corixa, Hamilton MT) (Cedarlane), 2% Alhydrogel (Cedarlane), or 2% Alhydrogel with MPL from *S. minnesota* R595 (Sigma Aldrich). Six week old, 20g female Balb/C mice, housed five to a cage, were used in all the experiments. The mice were ear-notched for individual identification. Pre-immunization blood samples were obtained from all mice via the jugular vein. The mice subsequently received two 0.1 mL anterior dorsal subcutaneous injections containing a total of 30 μg of Stx2 B subunit admixed with each of the adjuvants as per their manufacturer's instructions. One group of mice was sham immunized with pyrogen-free 0.9% NaCl irrigation solution, USP (Baxter Corporation, Toronto, Ontario, Canada). Alternatively, the mice were immunized with 30 μg of Stx2B-KLH conjugate or KLH admixed with RAS and TDM or 2% Alhydrogel. The mice were immunized at three week intervals, a maximum of three times. Seven days post immunization the mice were bled from the jugular vein to obtain post-immunization test sera.

3. 2. 4. Immunoblotting procedure to detect Stx2-specific antibodies in sera of immunized mice.

Conventional one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using Stx2 holotoxin in 15% separating gels and the A and B subunits were then electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). These immunoblots were blocked with a solution of 5% skim milk in phosphate buffered (pH 7.2) saline (PBS) and subsequently probed with the mouse sera diluted 2000 fold in PBS. These immunoblots were reacted with peroxidase-conjugated goat anti-mouse IgG (Sigma-Aldrich) diluted 2000 times. Immunoreactive bands were detected using the Enhanced Chemiluminescence (ECL™) detection kit (Amersham) with a 3 min exposure to Super RX Fuji medical X-ray film.

3. 2. 5. Analysis of mouse sera in the Ramos B cell cytotoxicity neutralization assay.

Five μL of mouse sera was preincubated with 0.05 ng of Stx2 holotoxin in saline for 20 minutes and then transferred to 0.5 mL of 5 x 10^5 Ramos B cells/mL of RPMI-1640. After 2 hours, the cells were washed two times by low speed centrifugation and re-suspended in fresh RPMI-1640 medium supplemented with 10% heat inactivated FBS. After a further 16 hour incubation at 37°C in an atmosphere of 5% CO_2/95% air, apoptotic Ramos cells were labeled with Annexin V-FITC (BD Pharmingen, Mississauga ON, Canada) and propidium iodide (Sigma Aldrich) as described by the supplier. Cell death was quantified by flow cytometry using a FACscan flow cytometer (Becton Dickinson, Mountain View CA).

3. 2. 6. Determination of mouse IgG1 and IgG2a titers by ELISA.

One hundred μL/well of a 2.5 μg/mL Stx2 PBS solution was incubated overnight at 4°C in 96 well EIA/RIA plates. The plates were then washed 5 times with PBS Tween (PBST) and blocked with 2.5% skim milk in PBST for two hours at 37°C. One hundred μL of serial dilutions of sera in PBST was added to the wells and the plates were incubated overnight at 4°C. The plates were next washed 5 times with PBST and 100 μL/well of a 1/2000 dilution of peroxidase-conjugated goat anti-mouse IgG1 or IgG2a antibodies (Southern Biotechnology, Birmingham, AL) was added for 2 hour at 37°C. The plates were washed 5 times with PBST and subsequently developed for 20 minutes with 100 μL/well of 10 mM citrate buffer, pH 4.2, 0.06% H_2O_2, 0.055% 2, 2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Boehringer Mannheim, Indianapolis, IN). The resulting absorbance data were recorded using a Spectramax 340 microtiter plate reader set at a wavelength of 410 nm. Background A_{410} readings obtained using sera from the sham immunized mice were subtracted from those obtained using sera from the immunized mice.

3. 2. 7. The murine Stx challenge model.

These experiments were conducted in a double-blind placebo-controlled manner. Fourteen days after receiving their last vaccine injection, the mice received a single anterior dorsal subcutaneous LD_{100} (0.2 ng/g body weight) injection of a cocktail consisting of Stx2 plus 7.5 μg Quil-A in 100 μl PBS. Beginning on the third day after initiating the Stx2 challenge the mice were monitored every 2 to 4 h and immediately euthanized by CO_2 asphyxia when signs of Shigatoxemia became apparent (Mulvey et al., 2003).

3. 2. 8. MTT proliferation assay.

Sub-confluent HRCE, RPTEC, NHMC or HMVEC-d monolayers were cultivated for 48 h in 96 well microtiter plates in the presence of serial dilutions of Stx2 or the recombinant Stx2 B subunit. A solution of 0.5 mg/mL MTT (3-[4, 5-dimethylthiazolyl-2]-2, 5-diphenyltetrazolium bromide, Sigma Aldrich, Oakville ON, Canada) in culture medium was then added to each well and the plates were incubated for 4 h at 37°C. Next, a solution of 10% SDS in 0.01 M HCl was added to the wells and the plates were incubated for an additional 18 h at 37°C. Formazan production was recorded using a Spectramax 340 microtiter plate reader set at a wavelength of 570 nm.

3. 3. Results

As in our previous rabbit study (Marcato et al., 2001), we detected an increase in the antigen-specific titer in all five mice injected with the recombinant Stx2 B subunit admixed with Quil A plus LPS (Figure 3-1). Both the IgG1 and IgG2a murine immunoglobulin subclasses were expressed but only after a third injection of antigen. In mice receiving three injections of the Stx2 B subunit admixed with Alhydrogel or RAS and TDM, with or without MPL, increases in the antigen-specific IgG1 titers were detected in most of the twenty immunized mice. By contrast, the antigen-specific IgG2a titer increased above the threshold in only three of these mice. None of the immunized mice in this pilot study produced

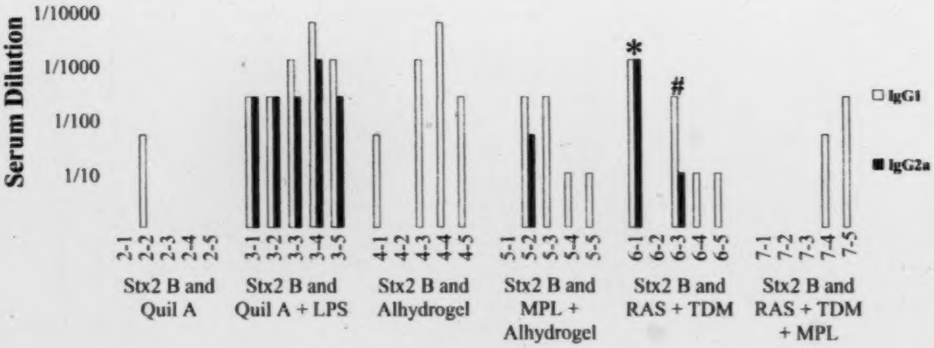

Figure 3-1. ELISA quantification of antigen-specific IgG1 and IgG2a titers in mouse sera after three injections of Stx2 B subunit admixed with the various adjuvant formulations. The titers are corrected for the background readings obtained using non-immune sera. The data represent the average of duplicate determinations. (*) mouse that survived and (#) mouse that showed increased survival, in a subsequent challenge with Stx2 holotoxin.

antibodies which reacted with the Stx2 B subunit in immunoblots or neutralized Stx2 holotoxin in the Ramos cell apoptosis assay (data not shown).

In the Stx2 holotoxin challenge experiment, only two mice in the pilot study survived appreciably longer than the sham-immunized control animals. Although both of these survivors were in the RAS and TDM study group (Figure 3-2), this result was not significant ($p > 0.2$, Fisher exact test). Therefore, we observed no correlation between IgG1 and IgG2a titers, immunoblot reactivity, cytotoxicity neutralization or protection against a lethal injection of Stx2 holotoxin in the pilot vaccine study. When larger groups were used, however, we were able to show a statistically significant ($p = 0.006$, Fisher exact test) increase in the survival of mice immunized with the Stx2 B subunit-RAS-TDM formulation (Figure 3-3). Within this group IgG1 titers $\geq 1/2500$ correlated ($p = 0.041$, Fisher exact test) with survival (Figure 3-4). There was no correlation between IgG2a titers and survival.

With only two injections, the murine immune response to the Stx2 B subunit was improved by chemically coupling the antigen to KLH, a potent T-cell activator and carrier protein. Regardless of the adjuvant used, the Stx2 B subunit-KLH conjugate preparation elicited antigen-specific IgG1 titers that were as good, or better, than those achieved using three injections of the un-conjugated antigen (Figure 3-5). As well, the highest antigen-specific IgG1 and IgG2a titers and survival rates (Figure 3-6) were observed in the group of mice that received two

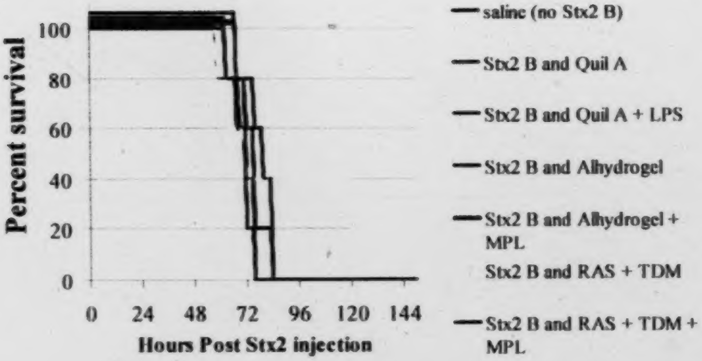

Figure 3-2. Survival plots for mice immunized with the Stx2 B subunit and various adjuvants and challenged with Stx2 holotoxin (n = 5 per group).

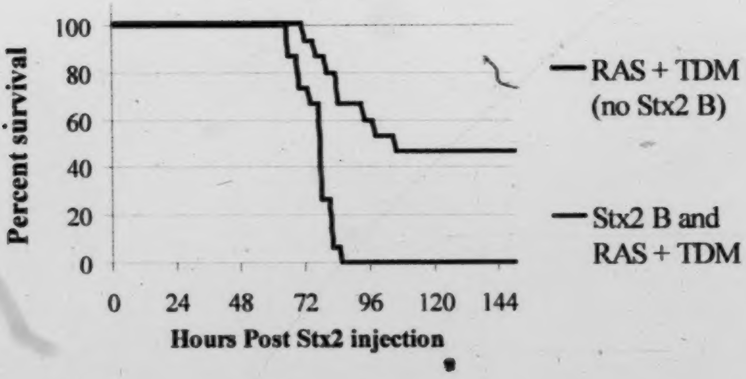

Figure 3-3. Survival plots for mice immunized with or without Stx2 B subunit and RAS plus TDM and challenged with Stx2 holotoxin (n = 15 per group, p = 0.006, Fisher exact test).

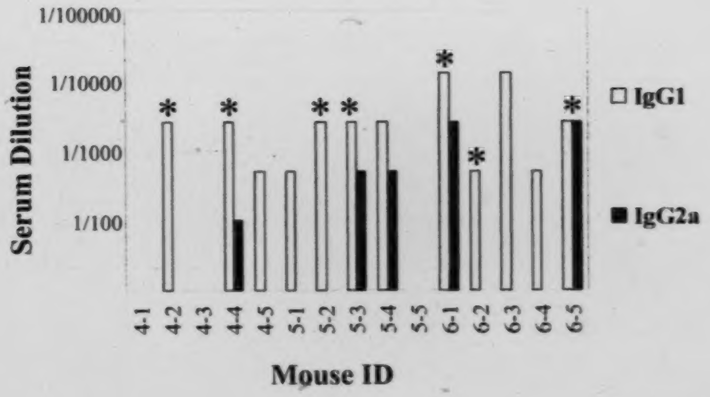

Figure 3-4. ELISA quantification of antigen-specific IgG1 and IgG2a titers in mouse sera after three injections of Stx2 B subunit admixed with RAS plus TDM. The titers are corrected for the background readings obtained using non-immune sera. The data represent the average of duplicate determinations. (*) mice that survived a subsequent challenge with Stx2 holotoxin.

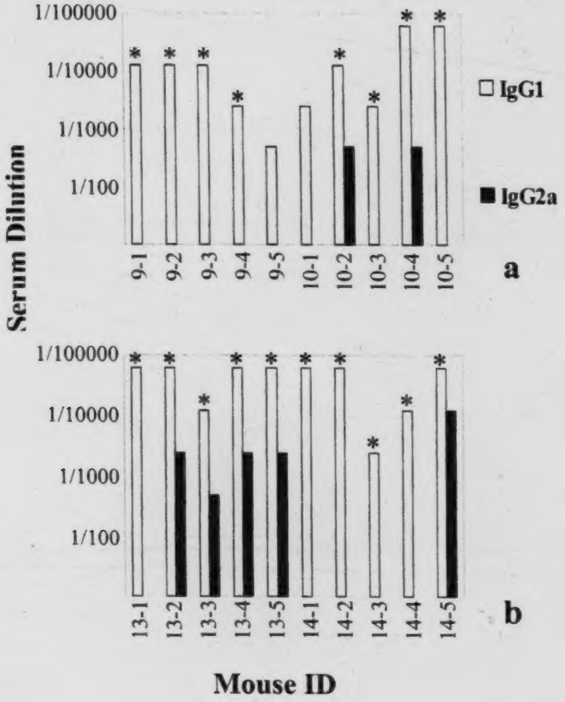

Figure 3-5. ELISA quantification of antigen-specific IgG1 and IgG2a titers in mouse sera after two injections of Stx2 B subunit-KLH conjugate admixed with Alhydrogel (**a**) or RAS-TDM (**b**). The titers are corrected for the background readings obtained using the KLH immunized mice. The data represent the average of duplicate determinations. (*) mice that survived a subsequent challenge with Stx2 holotoxin.

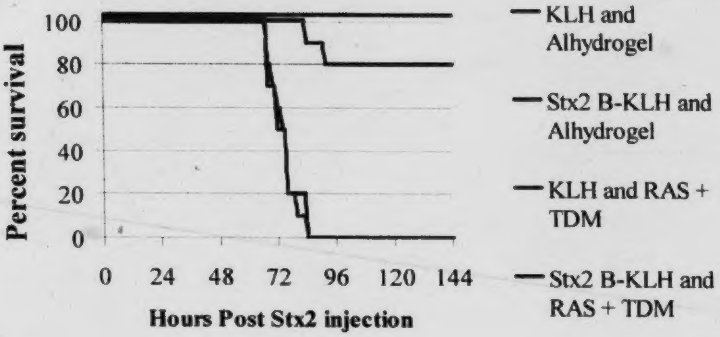

Figure 3-6. Survival plots for mice immunized with the Stx2 B subunit-KLH conjugate admixed with RAS plus TDM or Alhydrogel and challenged with Stx2 holotoxin (n = 10 per group, $p < 0.001$, Fisher exact test).

injections of the Stx2 B subunit-KLH conjugate in combination with RAS plus TDM. We also observed that the sera from seven of these mice neutralized Stx2 holotoxin in the Ramos cell apoptosis assay (Figure 3-7). However, this was not necessarily predictive of survival in the mouse challenge model since sera from protected mice immunized with the Stx2 B subunit-KLH conjugate in Alhydrogel failed to neutralize the apoptogenic activity of Stx2 in Ramos B cells. None of the sera from mice immunized with the Stx2 B subunit-KLH conjugate vaccine, regardless of the adjuvant used, reacted with the Stx2 B subunit in immunoblots (data not shown).

Several reports indicate that, at high concentrations, the Stx1 B subunit induces apoptosis in certain tissue culture cell lines (Arab et al., 1998; Mangeney et al., 1993; Pijpers et al., 2001). This has created safety concerns about using the recombinant Stx B subunit in a human EHEC vaccine. However, we have found that neither the Stx1 B or Stx2 B subunit induces apoptosis in Burkitt's lymphoma B cells (Marcato et al., 2002, 2003a). Several previous reports indicated that renal proximal tubular epithelial cells, renal epithelial cells and mesangial cells are highly sensitive to the cytotoxic effects of Stx1 and/or Stx2 holotoxin (Hughes et al., 1998; Hughes et al., 2000; Kiyokawa et al., 1998; Simon et al., 1998; Taguchi et al., 1998; Van Setten et al., 1997; Williams et al., 1999). HMVEC-d cells have also been used to study the pathogenic role of Stx1 and Stx2 in HUS (Ohmi et al., 1998). Here we directly compared the relative cytotoxicity of Stx2 holotoxin in these various primary tissue culture cell lines with that of the cloned

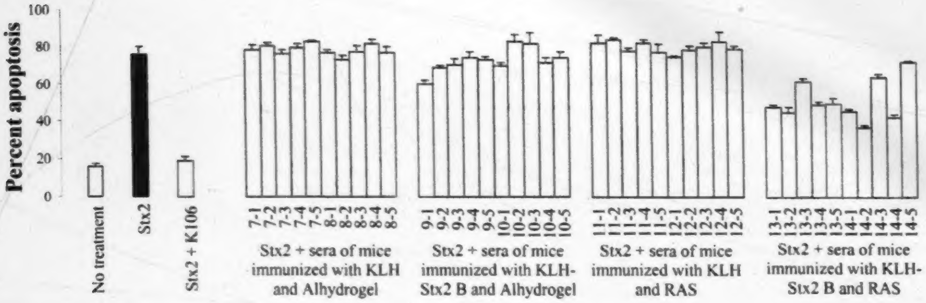

Figure 3-7. Cytotoxicity (Ramos cell apoptosis) neutralizing activity of sera obtained from mice immunized with the Stx2 B subunit-KLH conjugate admixed with RAS plus TDM or Alhydrogel. The experiments were performed in triplicate and the error bars represent the standard errors of the means. The white bars represent serum samples that significantly reduced Stx2 toxicity ($p \leq 0.05$, Student t-test). K106 is serum from a rabbit immunized with Stx2 B subunit and subsequently survived a Stx2 challenge (Marcato et al., 2001).

Stx2 B subunit preparation. Whereas Stx2 holotoxin demonstrated various degrees of cytotoxicity in these primary cells, the Stx2 B subunit preparation did not display any evidence of cytotoxicity, even at a concentration of 100 μg/mL (Figure 3-8).

3. 4. Discussion

Advocates for the development of a human EHEC vaccine have suggested using bacterial surface antigens or the Shiga toxins (Uchida, 2003; Yamasaki, 2002). Live attenuated or formaldehyde killed whole cell lysate vaccines against some EHEC serotypes O157:H7, O26:H11, O139:H1 have been also proposed (Yamasaki, 2002). While there is no denying the effectiveness of such vaccines at inducing a protective immune response, modern vaccines are typically acellular and based on highly purified more chemically defined bacterial antigens. O-specific polysaccharide-based vaccines have been designed that induce an anti-LPS immune response thereby preventing colonization (Conlan et al., 2000; Conlan et al., 1999; Konadu et al., 1999; Konadu et al., 1994; Konadu et al., 1998; Tana et al., 2003; Wang et al., 1999). However, O-polysaccharide-based vaccines suffer from the limitation that the immunity they induce is serotype-specific and a large number of EHEC serotypes have been implicated in human cases of HC and HUS.

Rabbits or mice immunized with the recombinant Stx1 B subunit developed neutralizing antibodies and a protected against subsequent Stx1 holotoxin

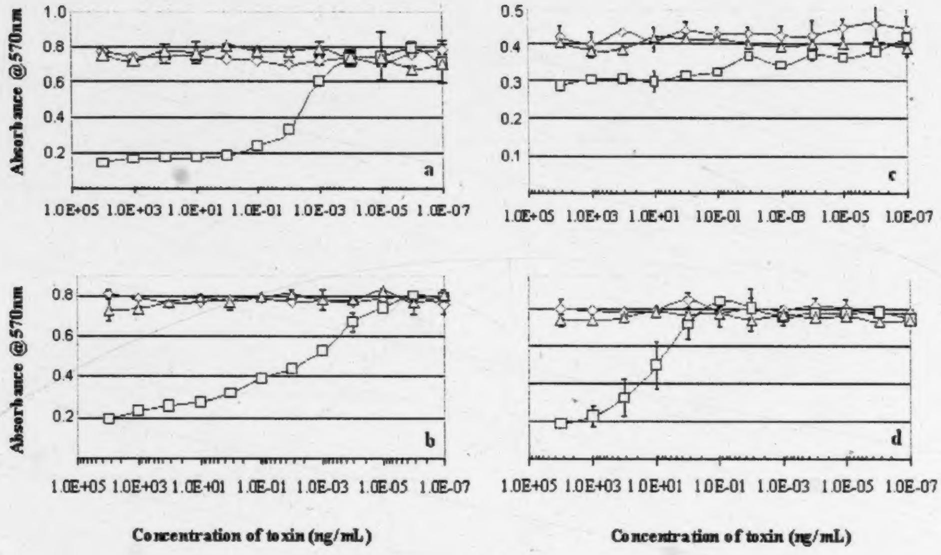

Figure 3-8. Cytotoxicity of Stx2 holotoxins and recombinant Stx2 B subunits as determined in cultured primary HRCE (**a**), RPTEC (**b**), NHMC (**c**), and HMVEC-d (**d**) cells. The sub-confluent monolayers were incubated with the toxins for 48 h and cell viability was then determined using the MTT proliferation assay. The error bars represent standard deviations (n = 3). Stx2 holotoxin (open squares) Stx2 B subunit (open triangles) no additions (open diamonds).

challenge (Acheson et al., 1996a; Boyd et al., 1991; Butterton et al., 1997; Byun et al., 2001). Oral immunization of mice with recombinant Stx1 B or Stx2 B subunits combined with cholera toxin as an adjuvant expressed anti-Stx1 B subunit antibodies, but failed to express an anti-Stx2 B subunit-specific response (Byun et al., 2001). However, given the importance of Stx2 in EHEC disease, and the observation that antibodies to the Stx2 B subunit are indicative of protection in a population (Ludwig et al., 2002), a protective Stx2 immune response would be desirable in a Stx-based EHEC vaccine. By chemically crosslinking the recombinant Stx2 B subunit to KLH and selecting the optimal adjuvant, we have demonstrated that it is possible to overcome the hypo-immunogenicity of the Stx2 B subunit and induce a protective immune response in mice.

The adjuvants selected for this study are representative of three classes of adjuvants. RAS is an oil emulsion, Alhydrogel is an aluminum salt, and Quil-A is a saponin-based adjuvant (Allison, 1998). MPL and LPS are bacterial products and represent a class of immunomodulators that can augment the immune response and/or change the antibody isotype induced (McCluskie and Weeratna, 2001). MPL is detoxified LPS purified from *S. Minnesota*. It is an experimental adjuvant currently in human clinical trials (Evans et al., 2003). Quil A is widely used in veterinary vaccines and more defined formulations, such as QS21, have been advocated for human use (Heath et al., 2003; Slovin and Scher, 1999). Quil A was used in this present study to compare results with those obtained in our previous Stx2 B subunit rabbit immunization study (Marcato et al., 2001). In mice, we now show similarly that the Stx2 B subunit admixed with Quil A alone was ineffective at inducing an effective immune response (Figure 3-1). The addition of LPS to the formulation significantly augmented both IgG1 and IgG2a antigen-specific titers that are indicative of a humoral and cell-mediated response, respectively. Generation of an IgG1 antibody response is dependent on antibody class switching induced by the profile of cytokines produced by Th2 cells whereas, generation of an IgG2a antibody response is dependent on the cytokines produced by Th1 cells. A strong humoral response would be desirable in preventing Shigatoxemia. However, in the challenge experiment, these animals fared no better than sham-immunized control mice.

RAS is a synthetic adjuvant that substitutes highly purified bacterial components for the killed *M. tuberculosis* in Freud's Complete Adjuvant (FCA). In this case, TDM and MPL are incorporated into the emulsion. Using purified bacterial products greatly reduces the reactogenic side affects associated with FCA, while still retaining robust immunostimulatory properties. While the overall IgG titers induced by the Stx2 B subunit-RAS and TDM formulation were not greater than those produced by the Stx2 B subunit admixed with Quil A and LPS, this formulation induced an immune response which provided less than 50% protection in the Stx2 challenge experiment (Figures 3-1 and 3-4). Survival of mice immunized with the Stx2 B subunit and RAS correlated with higher IgG1 titers and therefore a desirable humoral immune response (Figure 3-4). When MPL was added to RAS, this resulted in lower IgG titers and no increased survival in the challenge experiment (Figures 3-1 and 3-2). Therefore, MPL was apparently not beneficial in mice immunized with Stx2 B subunit in combination with RAS.

The aluminum hydroxide gel, Alhydrogel, and other aluminum salts are the only adjuvants currently used in human vaccines. Aluminum hydroxides are known to induce an IgG1 and therefore the desired humoral immune response (Allison, 1998). Even though the Stx2 B subunit combined with Alhydrogel induced IgG1 titers in immunized mice which were similar to those induced by the Stx2 B subunit combined with RAS, this immune response was not protective (Figure 3-1). Again, IgG titers alone are not predictive of vaccine efficacy.

KLH is approved for human use and has been successfully used as carrier for weak antigens in many applications (Musselli et al., 2001; Theilacker et al., 2003; Wang et al., 2000). After two injections of the Stx2 B subunit-KLH conjugate, the IgG1 titers were at least as high as those in mice immunized three times the un-conjugated Stx2 B subunit (Figures 3-1 and 3-5). Evidence of the improved immune response was also seen in the Stx2 cytotoxicity neutralization assays (Figure 3-7) and challenge experiment (Figure 3-8). With the Stx2 B subunit-KLH conjugate, both RAS plus TDM and Alhydrogel were effective at inducing an anti-Stx2 B subunit immune response that was protective in the challenge experiment. Again, IgG1 titers that are indicative of a humoral response correlated with survival in these challenge experiments (Figure 3-5 and 3-6).

By using adjuvants and conjugation to KLH, we have overcome difficulties associated with inducing a protective immune response to the recombinant Stx2 B subunit (Acheson et al., 1995; Byun et al., 2001; Marcato et al., 2001). These results contribute to the development of a Stx2 B subunit-based EHEC vaccine as we effectively protected mice from the lethal effects of Stx2 using an atoxic endotoxin free antigen with a human approved adjuvant and carrier protein.

Chapter 4

Serum amyloid P component binding to Shiga toxin 2 requires both A subunit and B pentamer

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K. Vander Helm performed the solid-phase binding and competitive binding assays in this chapter and is acknowledged in the Infection and Immunity version of this chapter as contributing equally. G. L. Mulvey contributed intellectually.

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In this chapter, the recombinant Stx2 B subunit was used to investigate whether the interaction between Stx2 holotoxin and HuSAP was specific to the B pentamer. A positive result may raise concerns with the B subunit's acceptability as an acellular component of a human EHEC vaccine.

4. 1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) cause hemorrhagic colitis (HC) and, occasionally, hemolytic-uremic syndrome (HUS) in humans. EHEC express two Shiga toxins, Stx1 and Stx2, which contribute to this pathogenesis. Epidemiological evidence indicates that EHEC strains expressing both Stx1 and Stx2 or, Stx2 alone, are more likely to cause HUS than those expressing only Stx1 (Hashimoto et al., 1999; Kleanthous et al., 1990; Ostroff et al., 1989). Although the reasons for the greater toxicity of Stx2 remain unclear, they probably relate to a combination of factors (Head et al., 1991; Louise and Obrig, 1995; Rutjes et al., 2002; Suzuki et al., 2000; Thorpe et al., 2001; Yamasaki et al., 1999).

The cytotoxicity of Stx2, but not Stx1, is neutralized in cell culture assays by human serum amyloid P (HuSAP) component (Kimura et al., 2001). HuSAP is an acute phase protein found in the immunoglobulin-depleted fraction of normal human serum (10). T. Kimura and colleagues suggested that the association of Stx2 with HuSAP may also be relevant to EHEC pathogenicity (Kimura et al., 2001). The experiments described herein represent our efforts to further understand the association of Stx2 with HuSAP.

4. 2. Materials and Methods

4. 2. 1. Purification of Stx1 and Stx2 holotoxins and their respective A and B subunits.

Stx1, Stx2, and their recombinant B pentamers, were expressed in *E. coli* and affinity purified as described previously (Marcato et al., 2001; Mulvey et al., 1998) and in greater detail in chapter 2, in sections 2. 2. 3. and 2. 2. 4. The Stx1 and Stx2 B pentamers and their A subunits were also prepared from their respective holotoxins using the urea-HPLC method described by S. C. Head and colleagues (Head et al., 1991). The Stx holotoxins and hybrid holotoxins were functionally re-assembled by admixing and dialyzing these HPLC-separated components (Head et al., 1991).

4. 2. 2. Verocytotoxicity assays.

Verocytotoxicity assays were used to confirm that the re-assembled Stx holotoxins and hybrid holotoxins had regained their cytotoxic activity. These assays were performed as described previously (Armstrong et al., 1991). Briefly, serial dilutions of the toxins were added to confluent Vero cell monolayers in 96 well microtiter tissue culture plates and incubated for 48 hours. Subsequently the monolayers were fixed with methanol, stained with Giemsa and cytotoxicity correlated with absorbance at wavelength of 620nm using a Spectramax 340.

4. 2. 3. Solid-phase binding assays.

HuSAP (Calbiochem) was purchased from Cedarlane Laboratories Ltd. (Hornby, ON). The purity of the commercial HuSAP preparation was confirmed by routine SDS-PAGE analysis under reducing conditions which, upon Coomassie blue staining, revealed a single band indicative of a protein with an apparent molecular weight of approximately 25K. HuSAP antiserum was obtained from Biogenesis Inc. (Kingston, NH). Microtiter plates from Fisher (Costar EIA/RIA ELISA) were coated for 18 h at 4°C with purified Stx1 or Stx2 holotoxins, or their respective separated subunits, at a concentration of 1 μg/mL in phosphate-buffered (pH 7.2) physiological saline (PBS). The wells were then thoroughly washed with 1% (w/v) bovine serum albumin (BSA) in PBS to remove unbound proteins and incubated for 1 h at 37°C with blocking solution (3 % w/v BSA in PBS) (Kimura et al., 2001). After removing the BSA blocking solution, HuSAP, serially diluted in 3% BSA-PBS, was added to the appropriate wells. The plates were incubated for 1 h at 37°C, washed with 1% BSA-PBS solution, and anti-HuSAP serum, diluted 4000 fold in 3% BSA-PBS, was then added for 1 h at 37°C. The plates were again washed with 1% BSA-PBS prior to adding horse radish peroxidase (HRP)-conjugated goat anti-rabbit IgG serum (diluted 2000 fold in 3% BSA-PBS). The chromogenic substrate, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS, Roche Diagnostics, Laval, QC) was added after the final washing step. Color development was allowed to proceed for 20 min. The results were then recorded using a Spectramax 340 microtiter plate reader at a wavelength of 405 nm.

4. 2. 4. The competitive solid-phase binding inhibition assays.

The competitive binding inhibition assays were performed using Stx2 holotoxin-coated microtiter plates described in the previous section. The competitive inhibitors were serially diluted in 3% BSA-PBS. Serial dilutions of the soluble Stx holotoxins or their HPLC-separated components were prepared in 3% BSA-PBS containing 0.1 μg/mL HuSAP in micro-centrifuge tubes. These samples were incubated for 1 h at 37°C before adding them to the appropriate wells in the Stx2-coated microtiter plates. The plates were incubated for an additional 1 h at 37°C and, after washing, bound HuSAP was detected as described above. Alternately, serial dilutions of the Daisy Gb_3 receptor analog (provided by Dr. David R. Bundle, Department of Chemistry, University of Alberta, Edmonton, Canada) (Mulvey et al., 2003) were added to the appropriate wells and the plates were incubated for 1 h at 37°C. The plates were then washed with 1% BSA-PBS and a solution containing 1 μg/mL of HuSAP was added for 1 h at 37°C. Once again, the plates were washed with 1% BSA-PBS and HuSAP binding was detected using primary and secondary antibodies as described in the previous section.

4. 2. 5. Ramos cell HuSAP Stx neutralization Assay.

Ramos cells (5 x 10^5 cells/mL) were treated, as described previously (Marcato et al., 2002), for two hours with Stx1, Stx2, Stx 1A/2B, or Stx 2A/1B hybrids with varying concentrations of HuSAP. The cells were washed with RPMI medium to remove excess unbound toxin, and incubated for an additional 16 hours at 37°C. The cells were then labeled with Annexin V-FITC (BD Pharmingen, Mississauga ON) and propidium iodide (Sigma Aldrich) as instructed by the manufacturer. Cell death was quantified by flow cytometry using a FACscan flow cytometer (Becton Dickinson, Mountain View CA).

4. 3. Results and Discussion

The data in Figure 4-1 duplicate the results originally published by T. Kimura and colleagues and demonstrate that native Stx2, but not Stx1 holotoxin, binds HuSAP in a solid-phase binding assay (Kimura et al., 2001). In an extension of these experiments by T. Kimura and colleagues, we now show, also in Figure 4-1, that HuSAP did not bind to any of the separated Stx1 or Stx2 subunits in this same assay (Kimura et al., 2001). However, HuSAP binding did occur when Stx2 holotoxin that had been re-assembled from its separated A subunit and B pentamer was used in the assay. By contrast, only minimal HuSAP binding was detected to the hybrid holotoxin composed of the Stx1 A subunit and Stx2 B pentamer (Figure 4-1, insert). HuSAP binding was not detected when the hybrid holotoxin composed of the Stx2 A subunit and Stx1 B pentamer was used in these

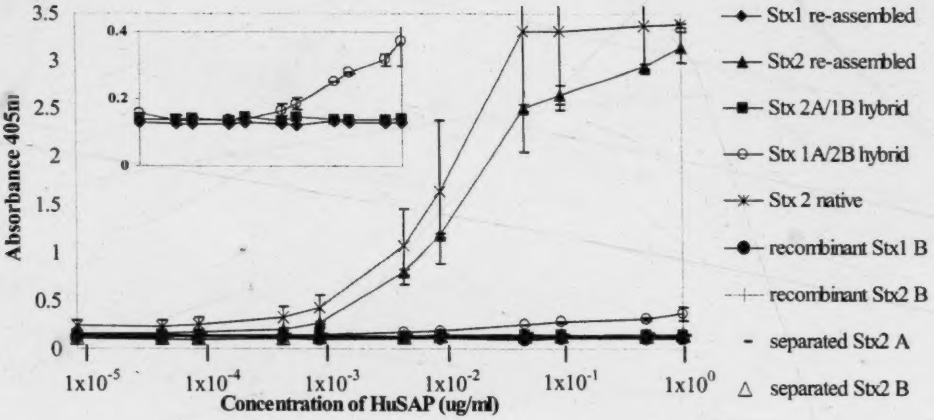

Figure 4-1. HuSAP binding to immobilized Stx proteins. The data represent the average (n = 3) for each HuSAP dilution and the error bars represent the standard deviations. Absence of error bars indicate that these were too small to appear on the graph. The insert displays the data for the Stx1 holotoxin and the re-assembled hybrid toxins plotted with an expanded Y axis.

experiments. The biological activity of all these re-assembled holotoxins was confirmed in the Verocytotoxicity assay. These data (Figure 4-2) demonstrated that the separated Stx subunits had indeed re-assembled into fully functional holotoxins.

We considered the possibility that HuSAP may have been unable to access its binding sites on the immobilized separated Stx subunits. To test this, we performed competitive binding inhibition experiments using Stx holotoxins, separated Stx subunits, and hybrid holotoxins in solution-based binding inhibition experiments. In these assays, the hybrid holotoxin composed of the Stx1 A subunit and Stx2 B pentamer and the HPLC-separated Stx2 B pentamer competitively inhibited HuSAP binding to immobilized Stx2 at 10 and 100 fold higher concentrations respectively versus the Stx2 control or re-assembled Stx2 holotoxin (Figure 4-3). These results are consistent with the data in Figure 4-4, illustrating that HuSAP neutralizes Stx2 and, to a lesser extent, the Stx 1A/2B hybrid toxin in the Ramos cell apoptosis assay.

The simplest interpretation of these collective results is that, whereas the B pentamer appears to make the more significant contribution, HuSAP binding requires domains from both the Stx2 A subunit and the Stx2 B pentamer.

We rejected the notion that instability of the Stx2 B pentamer, absent the A subunit, contributed to the toxin's reduced ability to bind HuSAP because we previously demonstrated (Marcato et al., 2001), and confirmed herein by HPLC size exclusion chromatography (data not shown), that the recombinant Stx2 B

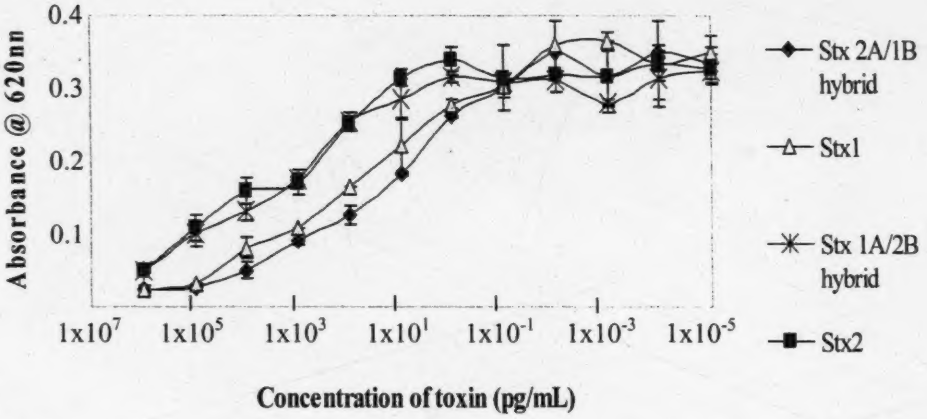

Figure 4-2. Cytotoxic activities of re-assembled Stx1, Stx2 and hybrid holotoxins (Stx1A/Stx2B and Stx2A/Stx1B) in Vero cells (n = 2), and the error bars represent standard errors).

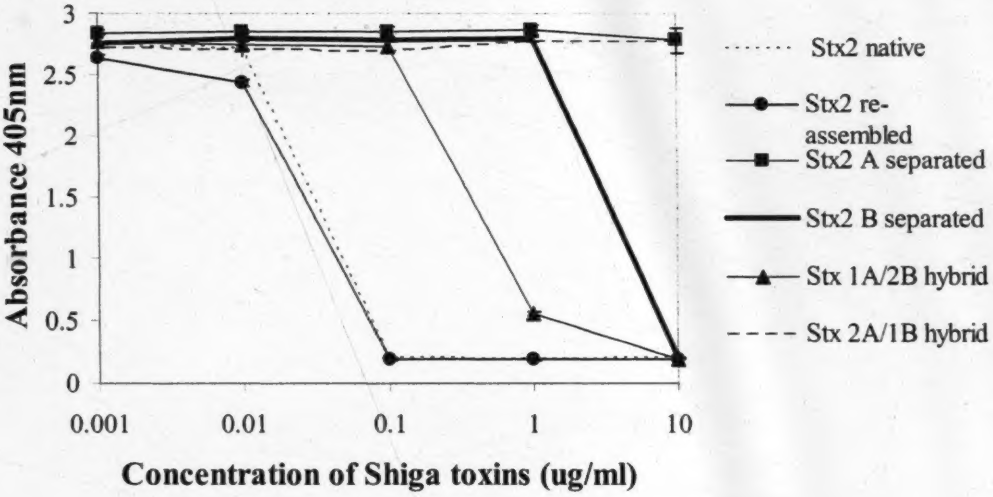

Figure 4-3. HuSAP competitive binding to immobilized Stx2 in the presence of increasing concentration of soluble Stx proteins. The data represent the average (n = 3) for each dilution and the error bars represent standard deviations. Absence of error bars indicate that these were too small to appear on the graph.

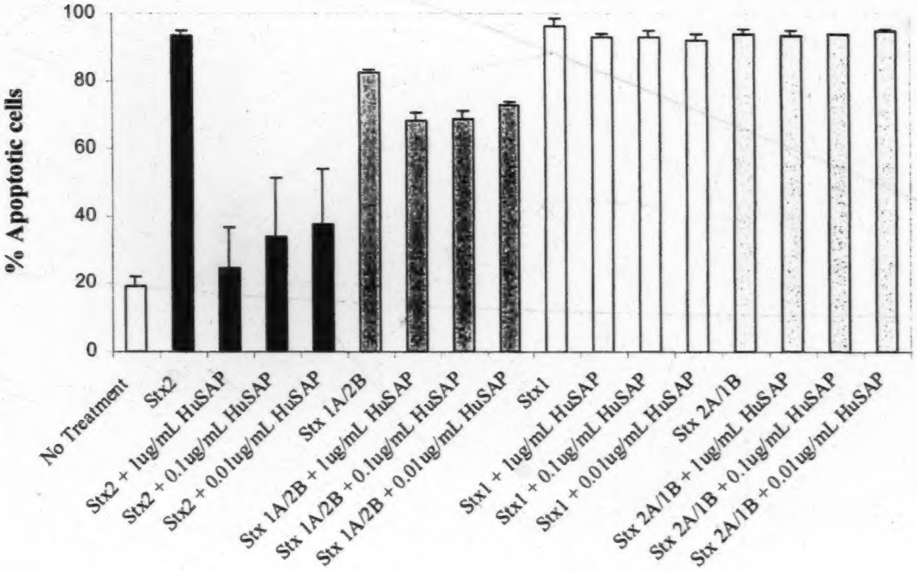

Figure 4-4. HuSAP neutralization of apoptosis induced by Stx holotoxins and re-assembled hybrid toxins in Ramos B cells. The data represent the average (n = 6 for the no treatment and Stx2 with or without HuSAP, and n = 3 for the remaining inhibitors) and the error bars represent the standard deviations. There is statistically significant difference between the Stx2 treated cells and the Stx2 with HuSAP treated cells, and the Stx1A/2B treated cells and the Stx1A/2B with HuSAP treated cells ($p < 0.001$, Student t test).

subunits remained stable as pentamers in solution. Absent any X-ray crystallographic evidence to the contrary, it is possible, however, that the tertiary conformation of the Stx2 B pentamer is slightly altered when it associates with the A subunit, thereby exposing cryptic HuSAP binding sites in the Stx2 B subunits.

The globotriaosylceramide receptor-based polyvalent inhibitor, called Daisy (Mulvey et al., 2003), was also used in the HuSAP competitive binding inhibition assays. We previously reported 50% binding inhibition constants (IC_{50}s) for Daisy in the sub-micromolar range (Mulvey et al., 2003). Given its demonstrated high avidity for the Stx2 B pentamer, Daisy should have inhibited HuSAP binding to Stx2 if these two ligands indeed competed for the same binding sites in the toxin. However, even at the highest concentration that could be achieved (10 mM), Daisy failed to competitively inhibit HuSAP binding to Stx2 holotoxin (data not shown).

At the present time, it is uncertain if Stx2 binding to HuSAP is at all relevant to EHEC pathogenesis. It is possible that the neutralizing function of HuSAP might prevent the majority of EHEC patients from developing HUS (Kimura et al., 2001). In this instance, the risk of HUS may be greater in EHEC-infected individuals expressing, for one reason or another, abnormally low serum concentrations of HuSAP. However, in their more recent report (Kimura et al., 2003), Kimura and colleagues failed to obtain *in vivo* evidence for this intriguing hypothesis.

S. R. Nelson and colleagues previously reported that the serum concentration of SAP ranges from 0.19 to 0.26 μM in healthy human adults and is relatively unaffected by an individual's pathophysiological status (Nelson et al., 1991). Assuming that a toxic concentration of Stx2 in humans is probably 2.6 pM (0.2 μg/kg body weight) (Mulvey et al., 2003), the serum concentration of SAP would be at least 10^4 to 10^5 times greater than any circulating Stx2 in an EHEC-infected subject. If so, any decrease in SAP concentration would have to be very dramatic if it was to influence whether or not an EHEC-infected subject developed HUS. Extrapolating these same arguments to children, who are more at risk for developing the Stx-mediated systemic complications on an EHEC infection, may not be wise, however, because the serum concentration of SAP is unknown in these subjects and may well be much lower than that in adults. Our continuing studies into the biochemical basis for, and biological consequences of, Stx2 binding to HuSAP are now focused on resolving these important issues.

Chapter 5

General Discussion and Future Directions

5. 1. Comparison of Shiga toxins

In this thesis, the *stx1b* leader sequence was utilized to overcome difficulties associated with expressing recombinant Stx2 B pentamers. While the possibility of using the cloned Stx2 B subunit as an acellular component in a human vaccine is a worthwhile endeavor, it is not its only use. Previously, researchers interested in determining toxin related pathogenesis could only utilize the readily available cloned Stx1 B subunit as a tool. While this has advanced our understanding of the pathogenic role of the Shiga toxins, it has also inevitably resulted in generalized conclusions regarding the receptor binding component of both Stx1 and Stx2.

It has become increasingly evident that Stx2 plays a much more critical role in the manifestations of the disease. The availability of the cloned Stx2 B subunit as an experimental tool has aided in the study of the increased toxicity of Stx2 in humans. The B pentamer is especially relevant, as tissue-targeting and receptor binding have been identified as key determining toxicity factors (Head et al., 1991; Nelson et al., 1994; Rutjes et al., 2002). A weakness in many Stx pathogenesis studies is the sole use of one toxin or the other and the lack of direct comparison between the two. The conclusions on EHEC pathogenesis in relation to Stx would be much more meaningful if Stx1 and Stx2 were compared in the same study. One such example is with the Stx2 binding to HuSAP (Kimura et al., 2001). Absent the finding that Stx1 conversely does not interact with HuSAP, the HuSAP interaction with Stx2 would be much less intriguing. The contrasting properties of the two toxins raises questions regarding their unique roles in EHEC pathogenesis. The conclusions that can be made from experiments comparing Stx1 and Stx2 are more relevant to the disease than when only one of the toxins is studied.

5. 2. HuSAP interaction with Stx2 and the immune response

A natural function of SAP is to bind extracellular DNA in chromatin that has been released by apoptosis or necrosis, stabilize it from degradation, and prevent autoimmunity from occurring (Bickerstaff et al., 1999; Breathnach et al., 1989; Pepys and Butler, 1987). Therefore, it is possible that binding of HuSAP to Stx2 might also prevent an anti-Stx2 immune response from developing. This may contribute to the associated expression of Stx2 by EHEC and a more severe course of illness.

Not only does HuSAP binding to Stx2 have implications with regards to EHEC pathogenesis, but this also has implications with respect to using the toxin and/or its subunits as acellular vaccine components. The interaction between Stx2 and SAP could interfere with effective immunization and also poses safety concerns because this interaction has uncertain physiological implications. Results in Chapter 4, demonstrate that maximal HuSAP interaction with Stx2 requires both the Stx2A subunit and B pentamer (Figure 4-1). In the future, X-ray crystallography studies could be used to confirm these findings and identify the specific epitopes of the A subunit and B pentamers that interact with HuSAP. In the meantime, however, the data tend to favor the use of the cloned B subunit as an immunization antigen, since, without the A subunit, the B subunit had minimal HuSAP binding activity (Figure 4-3). The lack of *in vitro* binding suggests that it would be unlikely that *in vivo*, HuSAP would interfere with the immunological properties of the Stx2 B subunit. Therefore, using the Stx2 B subunit as an acellular vaccine component would have the advantage over using an enzymatically-inactivated Stx2 holotoxin that might still interact with HuSAP.

In the future, it should be possible to confirm this *in vivo* by immunizing mice that are transgenic for HuSAP (Iwanaga et al., 1989). If the *in vitro* results (Chapter 4) are predictive of an *in vivo* interaction with HuSAP, then the Stx 2 B subunit is unlikely to interact with HuSAP in the mice, and the immunization results should be unaffected.

5. 3. HuSAP interaction with Stx2 and pathogenesis

Recent experiments using transgenic mice expressing HuSAP suggest a critical protective role of HuSAP in EHEC pathogenesis (G. D. Armstrong and M. B. Pepys, unpublished results). It is possible that a child expressing lower than normal amounts of HuSAP would be at a greater risk of developing EHEC-mediated HUS, than a child expressing an adequate amount. M. Bitzan and colleagues analyzed the sera obtained from asymptomatic individuals in three different age groups for Stx2 neutralizing activity (Bitzan et al., 1993a). The average neutralization titer observed in infants tended to be lower than that seen in older children and adults; however, the difference was not significant (Figure 5-1). Nevertheless, in each group the range of neutralizing titers was considerable. It is possible, therefore, that if individuals expressing lower concentrations of SAP were infected with EHEC, they might be at a greater risk for developing HUS. Future studies might involve quantifying the circulating levels of SAP in HUS patients in comparison to non-HUS age and sex matched controls. Such studies might reveal a possible therapeutic or prognostic role for HuSAP in EHEC-infected patients.

5. 4. Stx2 B subunit and the practicality of a human EHEC vaccine

The results presented in chapters 2 and 3 suggest that the Stx2 B subunit could be a potential candidate for a universal EHEC vaccine. The rabbit and mouse animal studies suggest that, with proper adjuvant formulation and conjugation, a vaccine composed of the cloned Stx2 B subunit could effectively protect against a lethal exposure to Stx2 holotoxin (Marcato et al., 2001). Further testing with an EHEC infection model such as the greyhound dog may be required to better test the efficacy of the Stx2 B subunit conjugate-based vaccine in preventing HUS and HC. In addition, it will be critical to evaluate the memory response to an immunization protocol involving the Stx2 B subunit. Ideally, protective immunity should last years, or, at least until a child is no longer in an age group

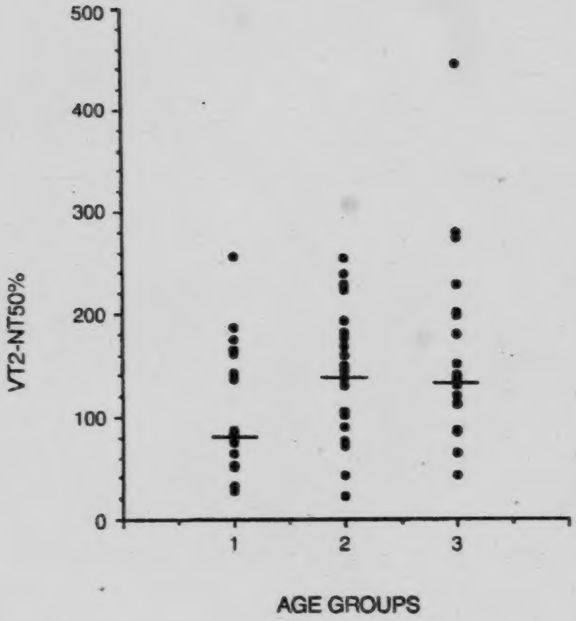

Figure 5-1. Median Stx2 (VT2) neutralization titers (NT50%) in sera from different age groups: (1) 5 to 22 months, (2) 2 to 10 years, (3) 18 to 65 years. Neutralization titers were determined to the highest dilution that protected 50% of the cells (Bitzan et al., 1993a).

associated with a greater risk of developing HUS. Other vaccine formulations, possibly incorporating the Stx1 B subunit with the Stx2 B-KLH conjugate, or perhaps conjugating it as well, might provide even greater protection against an EHEC infection.

Even if a Stx B subunit conjugate vaccine proves to be effective, developing an EHEC human vaccine may not be a realistic option, if practicality and marketability are also considered. As discussed in Chapter 1, the lack of treatment for EHEC-infected patients favors the option of immunization as a preventative measure. HUS is the leading cause of acute renal failure, however, the rate of HUS in North America and many other developed regions of the World is relatively low (Gianviti et al., 1994; Griffin and Tauxe, 1991; Martin et al., 1990; Rowe et al., 1991; Tarr and Hickman, 1987). In diseases where the incidence is low, the risk associated with vaccine-related adverse reactions is an argument against universal childhood immunization. However, certain countries such as Argentina with a high incicdence with 22 cases of HUS/100,000 children/year and 39% of children developing HC during the spring/summer months (Lopez et al., 1995), might represent a country where development of an EHEC vaccine might be a practical and useful goal. It may also be possible to immunoprophylactically vaccinate those individuals living in proximity an outbreak, thereby reducing their risk of developing the disease if they become infected. Another possibility might be to restrict vaccination to individuals in high-risk groups, such as children living in close proximity to intensive livestock operations (Beutin et al., 2000; Cadwgan et al., 2002). There are political and logistic concerns with these ideas. Finally, a safe universal vaccine for preventing the serious complications of EHEC infections might also be useful in the event these organisms ever find a use as agents of biological terror.

5. 5. Concluding Statement.

Given the current lack of practical therapeutic approaches for treatment of EHEC and methods of eliminating the organisms from the environment, the objective of my thesis research was to determine the feasibility of producing a safe and effective Stx-based vaccine for preventing the serious complications associated with EHEC infections in humans. The studies reported herein represent significant steps towards achieving that objective.

Chapter 6

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